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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A01N 63/02, A61K 38/10, 38/17, C07K 1/16, 4/12, 7/08, 14/435, 16/18, C12N 15/12, G01N 33/68

A1

(11) International Publication Number: **WO 95/23513**

(43) International Publication Date: 8 September 1995 (08.09.95)

(21) International Application Number: PCT/US95/02626

(22) International Filing Date: 2 March 1995 (02.03.95)

(30) Priority Data:
08/205,938 2 March 1994 (02.03.94) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/205,938 (CIP)
Filed on 2 March 1994 (02.03.94)

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(81) Designated States: AU, CA, JP, MX, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: APIDAECIN-TYPE PEPTIDE ANTIBIOTICS WITH IMPROVED ACTIVITIES AND/OR DIFFERENT ANTIBACTERIAL SPECTRUM

(57) Abstract

This invention provides a purified polypeptide having antibacterial activity comprising a first sequence Pro-Arg-Pro-Pro-His-Pro-Arg-X1, wherein X1 is Ile or Leu; and a third sequence X2-Pro-X3-X4-X5-Pro, wherein X2 is Arg or Lys, X3 is Thr, Gln or Arg, X4 is Tyr, Gln or Pro, and X5 is Val or Ala, the third sequence is N-terminal to the first sequence. This invention also provides a purified polypeptide having antibacterial activity comprising: a first sequence, at least seven amino acid residues are the same as Pro-Arg-Pro-Pro-His-Pro-Arg-X1, wherein X1 is Ile or Leu; a third sequence X2-Pro-X3-X4-X5-Pro, wherein X2 is Arg or Lys, X3 is Thr, Gln or Arg, X4 is Tyr, Gln or Pro, and X5 is Val or Ala, the third sequence is N-terminal to the first sequence; and a fourth sequence comprising at least five amino acid residues, at least one-third of the residues are Pro, the fourth sequence is N-terminal to the second sequence.

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APIDAECIN-TYPE PEPTIDE ANTIBIOTICS WITH IMPROVED ACTIVITIES AND/OR DIFFERENT ANTIBACTERIAL SPECTRUM

This application is a continuation-in-part of United States Application Serial No. 08/205,938, filed March 2, 1994, the contents of which are hereby incorporated by reference.

Throughout this application, various references are referred to within parenthesis. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of this application, preceding the sequence listing and the claims.

Background of the Invention

The following standard abbreviations are used throughout to refer to amino acids:

A	Ala	Alanine	M	Met	Methionine
C	Cys	Cysteine	N	Asn	Asparagine
D	Asp	Aspartic acid	P	Pro	Proline
E	Glu	Glutamic acid	Q	Gln	Glutamine
F	Phe	Phenylalanine	R	Arg	Arginine
G	Gly	Glycine	S	Ser	Serine
H	His	Histidine	T	Thr	Threonine
I	Ile	Isoleucine	V	Val	Valine
K	Lys	Lysine	W	Trp	Tryptophan
L	Leu	Leucine	Y	Tyr	Tyrosine

Other abbreviations include the following. PBS: phosphate buffered saline, BSA: bovine serum albumin, MeCN: acetonitrile, TFA: trifluoro acetic acid, Fmoc: 9-fluoroenylmethoxycarbonyl, PTH: phenyl thiohydantoin, RP-HPLC: reversed-phase high performance

liquid chromatography, MALDI-TOF: matrix-assisted laser-desorption ionization time-of-flight, MS: mass spectrometry, UV: ultra violet, ELISA: enzyme-linked immunosorbent assay, RT: room temperature, MIC: minimal inhibitory concentration, CFU: colony forming units, nt: not tested.

For more than a century, it has been known that bacteria are among the agents of disease. Disinfectants and antibiotics usually allow to contain unwanted microbial propagation, but not always. The exceptions thus necessitate a continuing search for novel antibiotics. During the last decade, many antibacterial peptides have been isolated from insects (for latest updates see Refs. 1 and 2, and references therein). While undoubtedly vital for the insects, to date, no strong efforts have been made to assess the prospects of clinical applications. Published accounts on screening insect peptides for activity against severe human pathogens or genuinely problematic, opportunistic bacteria are unavailable.

It is well-established however, that the overwhelming majority of antibacterial peptides, including the well-studied defensins, cecropins and magainins, function through a 'lytic/ionophoric' mechanism (3-11). Common theme among all 'lytic' peptides is a permeabilizing effect on bacterial cytoplasmic membranes. A cationic, amphipathic structure that enables formation of hydrophilic ion (proton) channels in a lipid bilayer (12) is fundamental to this activity; proton leakage causes dissipation of the membrane potential, required for many vital life processes, thus causing cell death (7,8,13,14). As perturbation of membranes by these peptides is not dependent on recognition of chiral molecules (15,16), amino acid substitutions that do not abrogate general amphipathic structure or basic net

charge are functionally tolerated (17,18).

Even if selected peptide antibiotics are of initial therapeutic efficacy, meaningful, long-term medical applications could only be considered after finding a way to target lethal activities to well-chosen groups of microbes, to cope with emerging resistance, and to create more stable, less antigenic and easier to produce analogs. In other words, will antibacterial peptides be amenable to specific manipulations (amino acid substitutions, deletions or truncations) leading to the synthesis of second generation chemotherapeutics, that are widely applicable and economically justified, or would offer a specific solution to recognized problems in antimicrobial therapy, such as treatment of infections in immuno-compromised hosts (19), resistent (20) or persistent strains (21), bacteremias (22) and previously unrecognized pathogens (23)? Rational modifications to existing peptides must be guided by the results of prior, detailed structure/function analyses. Obviously, short peptides offer distinct advantages for such studies. In addition, because of their generic lethal mechanism, 'lytic' peptides may not be particularly suited as backbone for those developments.

A unique peptide, 'apidaecin', has been isolated from honeybees (24). Apidaecin is small (18 unmodified, L-amino acids; 33% proline) and can easily be mass produced. The peptide inhibits viability of many gram negative bacteria in nanomolar doses; gram positives are unaffected. Lethal activity is near immediate and shown to be independent of the conventional 'lytic' mechanism (25). In addition, apidaecin-resistant mutants are of undiminished sensitivity to 'pore-forming' peptides and the D-enantiomer is devoid of antibacterial activities . The current model is that the antagonistic effects of apidaecin on bacteria involve stereoselective recognition

of chiral targets (25).

Understanding of the role played by each component amino acid in apidaecin might be obtained from exhaustive functional screening of synthetic analogs. This approach could easily develop into an unmanageable project as, for instance, producing combinatorial change in 6 positions would equal screening 64 million peptides, without any guarantee of an improved product. Although 'de-novo' peptide (6-7 residues) drug design using such combinatorial approaches (also known as peptide libraries) have been suggested (26), trying to accomplish this in the context of a 20 residue long peptide would be infinitely more difficult and labor intensive. Instead, nature (i.e. evolution) was turned to in order to understand structure / function of bioactive peptides. Insects are an ideal source of peptide to initiate such comparative structural analysis for reasons of (i) enormous evolutionary diversity (27,28), (ii) ease of rearing, handling, and inducing and harvesting peptide antibiotics, and (iii) their strong reliance on those peptides for immunity and, consequently, survival.

Summary of the Invention

This invention provides a purified polypeptide having antibacterial activity comprising a first sequence Pro-Arg-Pro-Pro-His-Pro-Arg-X1 (SEQ ID NO: 1), wherein X1 is Ile or Leu; and a third sequence X2-Pro-X3-X4-X5-Pro (SEQ ID NO: 2), wherein X2 is Arg or Lys, X3 is Thr, Gln or Arg, X4 is Tyr, Gln or Pro, and X5 is Val or Ala, the third sequence is N-terminal to the first sequence.

This invention provides a purified polypeptide having antibacterial activity comprising:

a first sequence, at least seven amino acid residues are the same as Pro-Arg-Pro-Pro-His-Pro-Arg-X1 (SEQ ID NO: 1), wherein X1 is Ile or Leu;

a third sequence X2-Pro-X3-X4-X5-Pro (SEQ ID NO: 2), wherein

X2 is Arg or Lys,

X3 is Thr, Gln or Arg,

X4 is Tyr, Gln or Pro, and

X5 is Val or Ala, the third sequence is N-terminal to the first sequence; and

a fourth sequence comprising at least five amino acid residues, at least one-third of the residues are Pro, the fourth sequence is N-terminal to the third sequence.

This invention provides DNA encoding a polypeptide as described above.

This invention provides a purified antibody capable of binding to a polypeptide as described above.

This invention provides a method for determining the presence of the polypeptide in a sample comprising: incubating the sample with the antibody described above, and detecting an antibody-antigen complex, thereby

determining the presence of the polypeptide in the sample.

This invention provides a method for inhibiting growth of a bacterium comprising administering to the bacterium a growth inhibiting effective concentration of a polypeptide as described above.

This invention provides a pharmaceutical composition comprising an antibacterial effective amount of a polypeptide as described above.

This invention provides a method for treating a subject infected with a bacterium comprising administering to the subject an antibacterial effective amount of a polypeptide as described above, thereby treating the subject.

This invention provides a method for obtaining a purified apidaecin-like polypeptide from a Hymenopteran insect comprising:

obtaining a sample of lymph from the insect;

treating the sample so as to obtain supernatant;

applying the supernatant to a reversed-phase high performance liquid chromatography column;

eluting from the column;

collecting the fractions eluted from the column; and

determining a fraction which contains the polypeptide, thereby obtaining the polypeptide from the insect.

Description of the Figures

Figure 1. Sequence alignment of apidaecin-type peptides. Numbering is based on the honeybee (Hb) sequences (24); naturally occurring isoforms are grouped per insect (separated by dotted lines). Dots (.) indicate that the residue in this position is identical to the one in the primary sequence (top line in each box) of that particular insect; dashes (-) represent a gap in the sequence, introduced for alignment purposes. Sequence similarities are boxed (solid lines) and the conserved consensus sequence is shown at the bottom (also boxed). [MH^+] is the theoretical molecular weight (average isotopic mass), calculated from the proposed sequence (using Procomp software); [*m/z*] values were experimentally obtained by MALDI-TOF mass spectrometric analysis. ND means not done; NA is not applicable (reason being that these peptides have never been observed in nature). Peptides Hb III was predicted from cDNA sequences (35); peptides Ho- and Cd2- were artificially lacking in GKP for comparative purposes.

Figure 2. Sequence alignment between hornet apidaecin and drosocin from *Drosophila*. Dashes (-) represent gaps, introduced for optimal alignment; identical residues are boxed. The drosocin structure is taken from Ref. 2; GalNAc-Gal stands for N-acetylgalactosamine-galactose (O-linked to Thr).

Detailed Description of the Invention

This invention provides a purified polypeptide having antibacterial activity comprising a first sequence Pro-Arg-Pro-Pro-His-Pro-Arg-X1 (SEQ ID NO: 1), wherein X1 is Ile or Leu; and a third sequence X2-Pro-X3-X4-X5-Pro (SEQ ID NO: 2), wherein X2 is Arg or Lys, X3 is Thr, Gln or Arg, X4 is Tyr, Gln or Pro, and X5 is Val or Ala, the third sequence is N-terminal to the first sequence.

In an embodiment the polypeptide has up to about thirty-five amino acid residues. In another embodiment the polypeptide has from about fourteen to about twenty-one amino acid residues.

While there is no limitation on the number of amino acid residues separating the first and third sequences, in a specific embodiment the third sequence is separated from the first sequence by up to two amino acid residues.

In an embodiment of the polypeptide, the third sequence is selected from the group consisting of:
Arg-Pro-Thr-Tyr-Val-Pro (SEQ ID NO: 3),
Arg-Pro-Gln-Gln-Val-Pro (SEQ ID NO: 4),
Arg-Pro-Arg-Pro-Ala-Pro (SEQ ID NO: 5), and
Lys-Pro-Arg-Pro-Ala-Pro (SEQ ID NO: 6).

This invention also provides the polypeptide, further comprising a fourth sequence selected from the group consisting of Gly-Lys-Pro and Asn-Lys-Pro, and Phe-Lys-Pro; the fourth sequence is N-terminal to the second sequence. While there is no limitation on the number of amino acid residues separating the third and fourth sequences, in a specific embodiment the fourth sequence is separated from the third sequence by up to two amino acid residues. In a specific embodiment, the third sequence is selected from the group consisting of:

Arg-Pro-Gln-Gln-Val-Pro (SEQ ID NO: 4),
Arg-Pro-Arg-Pro-Ala-Pro (SEQ ID NO: 5), and
Lys-Pro-Arg-Pro-Ala-Pro (SEQ ID NO: 6).

A purified polypeptide having antibacterial activity, comprising: a first sequence Pro-Arg-Pro-Pro-His-Pro-Arg-(Ile/Leu) (SEQ ID NO.:1); optionally, a second sequence immediately adjacent to the N-terminal amino acid residue of the first sequence, wherein the second sequence is selected from the group consisting of: Pro; Ala; Ile-Gln; and Ile-Lys; a third sequence immediately adjacent to the second sequence, or immediately adjacent to the N-terminal amino acid residue of the first sequence when the polypeptide does not contain a second sequence, wherein the third sequence has the following formula: X2-Pro-X3-X4-X5-Pro (SEQ ID NO: 2), wherein X2 is Arg or Lys; X3 is Thr, Gln or Arg; X4 is Tyr, Gln or Pro; and X5 is Val or Ala; and a fourth sequence immediately adjacent to the third sequence, wherein the fourth sequence is selected from the group consisting of: Asn; Gly-Lys-Pro; Ser-Asn-Lys-Pro (SEQ ID NO.:42); and Gly-Lys-Pro-(Asn/Ser) (SEQ ID NO.:43); wherein the fourth sequence is truncated by zero to four amino acid residues at its N-terminus; and wherein the number of amino acid residues in the polypeptide is the sum of the number of second sequence residues, the number of fourth sequence residues, and fourteen.

In an embodiment, the polypeptide comprises a sequence selected from the group consisting of:
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 7);
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Ala-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 8);
Gly-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 9);
Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu

(SEQ ID NO: 10);
Ser-Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 11);
Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 12);
Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13);
Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 14);
Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15);
Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 16);
Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17); and
Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 18).

In a more specific embodiment the polypeptide is selected from the group consisting of:
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 7);
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Ala-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 8);
Gly-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 9);
Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 10);
Ser-Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 11);
Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 12);
Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13);
Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 14);
Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-

Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15);
Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 16);
Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17); and
Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 18).

This invention further provides nucleic acid encoding the purified antibacterial polypeptide. Starting with an amino acid sequence, various nucleic acid molecules which encode the amino acid sequence can be generated based on the genetic code, which is known to those of skill in the art. The nucleic acid molecule can be either DNA or RNA, single stranded or double stranded. The single stranded molecule can be either the top (coding) or bottom (noncoding) strand. The single stranded nucleic acid molecule is useful as a probe. In a preferred embodiment this invention provides for a plasmid capable of expressing the polypeptide. The plasmid contains transcriptional and translational control sequences known to those of skill in the art.

This invention provides a purified polypeptide having antibacterial activity isolatable from an insect; the insect is selected from the group consisting of Sphecius speciosus, Vespula maculata, Vespula maculifrons, Paravespula germanica, and Coccygomimus disparis; the polypeptide comprising the sequence Pro-Arg-Pro-Pro-His-Pro-Arg. In an embodiment, the polypeptide is isolated from the lymph of the insect. In a preferred embodiment the polypeptide is isolated from an immuno-induced insect.

This invention also provides purified polypeptide having antibacterial activity isolatable from an insect; the insect is selected from the group consisting of Sphecius

speciosus, Vespula maculata, Vespula maculifrons, Paravespula germanica, and Coccygomimus disparis; the polypeptide characterized by binding to an anti-apidaecin antibody. In an embodiment, the polypeptide is isolated from the lymph of the insect. In a preferred embodiment the polypeptide is isolated from an immuno-induced insect.

This invention provides a purified antibody capable of binding to the polypeptide described herein. In an embodiment the antibody is a rabbit antibody. In one embodiment the antibody is a polyclonal antibody. In another embodiment the antibody is a monoclonal antibody.

This invention further provides a method for obtaining the purified antibody capable of binding to the polypeptide described herein comprising:
coupling an antigen selected from the group consisting of honeybee apidaecin and the polypeptide to a carrier protein;
immunizing a mammal with the coupled antigen; and
isolating the antibody from the mammal, thereby obtaining the purified antibody.

The coupling of an antigen which is poorly immunogenic to a carrier protein is known to those of skill in the art. Various carrier proteins are known to those of skill in the art. In an embodiment of the above method for obtaining the purified antibody capable of binding to the polypeptide described herein, the carrier protein is tuberculin purified protein derivative. In an embodiment the immunizing is immunizing by injecting. In a preferred embodiment the mammal is a rabbit.

This invention provides a method for determining the presence of the polypeptide in a sample comprising: incubating the sample with the antibody described above,

and detecting an antibody-antigen complex, thereby determining the presence of the polypeptide in the sample.

In the above method, the step of detecting the antibody-antigen complex can be performed in a number of ways known to one of skill in the art. In a preferred embodiment, the detecting comprises detecting by enzyme-linked immunoassay. In another embodiment, the detecting comprises radioimmunoassay.

This invention provides a method for inhibiting growth of a bacterium comprising administering to the bacterium a growth inhibiting effective concentration of the polypeptide described herein.

In an embodiment, this invention provides a method for inhibiting growth of a bacterium selected from the group consisting of:

Escherichia coli, Enterobacter cloacae, and Erwinia amylovora, Klebsiella pneumoniae, Salmonella typhimurium, Shigella dysenteriae, and Pseudomonas syringae;

comprising administering to the bacterium a growth inhibiting effective concentration of a polypeptide selected from the group consisting of:

Asn-Arg-Pro-Thr-Tyr-Val-Pro-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 7);

Asn-Arg-Pro-Thr-Tyr-Val-Pro-Ala-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 8);

Gly-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 9);

Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 10);

Ser-Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 11);

Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 12);

Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13);
Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 14);
Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15);
Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 16);
Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17); and
Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 18).

This invention also provides a method for inhibiting growth of an apidaecin resistant strain of Escherichia coli comprising administering a growth inhibiting effective concentration of a polypeptide selected from the group consisting of:
Gly-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 9);
Ser-Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 11);
Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 12);
Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13);
Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 14);
Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15);
Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 16);
Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17); and
Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 18).

This invention also provides a method for inhibiting growth of Morganella morganii comprising administering a growth inhibiting effective concentration of a polypeptide selected from the group consisting of:
Gly-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 9);
Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 10);
Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13);
Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 14);
Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15);
Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 16);
Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17); and
Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 18).

This invention also provides a method for inhibiting growth of Salmonella typhi comprising administering a growth inhibiting effective concentration of a polypeptide selected from the group consisting of:
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 7);
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Ala-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 8);
Gly-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 9);
Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 10);
Ser-Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 11);
Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 12);

Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13);
Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 14);
Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15);
Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 16); and
Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17).

This invention also provides a method for inhibiting growth of Yersinia enterocolitica comprising administering a growth inhibiting effective concentration of a polypeptide selected from the group consisting of:
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 7);
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Ala-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 8);
Gly-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 9);
Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 10);
Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 12); and
Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 14).

This invention also provides a method for inhibiting growth of Campylobacter jejuni or Helicobacter pylori comprising administering a growth inhibiting effective concentration of a polypeptide selected from the group consisting of:
Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13);
Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15);

Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 16);
Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17); and
Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 18).

This invention provides a method for inhibiting growth of Acinetobacter calcoaceticus comprising administering a growth inhibiting effective concentration of a polypeptide selected from the group consisting of:
Gly-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 9);
Ser-Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 11);
Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 12);
Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13);
Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 14);
Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15);
Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 16);
Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17); and
Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 18).

This invention also provides a method for inhibiting growth of Agrobacterium tumefaciens comprising administering a growth inhibiting effective concentration of a polypeptide selected from the group consisting of:
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 7);
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Ala-Pro-Arg-Pro-Pro-His-Pro-

Arg-Leu (SEQ ID NO: 8);
Gly-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 9);
Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 12);
Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13);
Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 14);
Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15);
Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 16);
Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17); and
Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 18).

This invention also provides a method for inhibiting growth of Francisella tularensis or Haemophilus influenzae comprising administering a growth inhibiting effective concentration of a polypeptide selected from the group consisting of:
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 7);
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Ala-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 8);
Gly-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 9);
Ser-Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 11);
Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 12);
Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13);
Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 14);

Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15);
Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 16);
Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17); and
Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 18).

This invention also provides a method for inhibiting growth of Legionella pneumophila comprising administering a growth inhibiting effective concentration of a polypeptide selected from the group consisting of:
Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13);
Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15);
Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 16);
Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17); and
Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 18).

This invention also provides a method for inhibiting growth of Rhizobium meliloti comprising administering a growth inhibiting effective concentration of a polypeptide selected from the group consisting of:
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 7);
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Ala-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 8);
Gly-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 9);
Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 10);
Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-

Pro-Arg-Leu (SEQ ID NO: 12);
Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13);
Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 14);
Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15);
Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 16);
Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17); and
Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 18).

This invention provides a pharmaceutical composition comprising an antibacterial effective amount of the polypeptide described herein and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are known to those with skill in the art. Examples include buffered saline solution and starch. In specific embodiments the pharmaceutical composition is a liquid, a cream, or a solid such as a tablet.

This invention provides a method for treating a subject infected with a bacterium comprising administering to the subject an antibacterial effective amount of a polypeptide described above, thereby treating the subject. In an embodiment, the subject is a mammalian subject. In a more specific embodiment the subject is a human subject. The polypeptide is administered according to techniques known to those of skill in the art, including orally, parenterally, intraperitoneally, by intramuscular injection, by intravenous injection, or topically.

This invention provides a method for obtaining a purified apidaecin-like polypeptide from a Hymenopteran insect

comprising: obtaining a sample of lymph from the insect; treating the sample so as to obtain supernatant; applying the supernatant to a reversed-phase high performance liquid chromatography column; eluting from the column; collecting the fractions eluted from the column; and determining a fraction which contains the polypeptide, thereby obtaining the polypeptide from the insect.

In a preferred embodiment of the method for obtaining a purified apidaecin-like polypeptide from a Hymenopteran insect, the obtaining a sample of lymph comprises puncturing the abdomen of the insect and collecting the hemolymph. In an embodiment the treating comprises centrifuging. In a preferred embodiment wherein the eluting is eluting with an ascending acetonitrile gradient. In an embodiment the determining is determining by enzyme-linked immunoassay, preferably comprising a first antibody being an anti-honeybee-apidaecin antibody. In a preferred embodiment the first antibody is a polyclonal antibody.

A preferred embodiment further comprises, before obtaining a sample of insect lymph, immuno-inducing the insect. In an embodiment the immuno-inducing comprises infecting the insect with an immune-response-inducing effective amount of bacterium, such as E. coli.

This invention provides a purified polypeptide having antibacterial activity comprising: a first sequence, at least seven amino acid residues are the same as Pro-Arg-Pro-Pro-His-Pro-Arg-X1 (SEQ ID NO: 1), wherein X1 is Ile or Leu; a third sequence X2-Pro-X3-X4-X5-Pro (SEQ ID NO: 2), wherein X2 is Arg or Lys, X3 is Thr, Gln or Arg, X4 is Tyr, Gln or Pro, and X5 is Val or Ala, the third sequence is N-terminal to the first sequence; and a fourth sequence comprising at least five amino acid residues, at least one-third of the residues are Pro, the

fourth sequence is N-terminal to the third sequence.

In an embodiment the polypeptide has up to about thirty-five amino acid residues.

While there is no limitation on the number of amino acid residues separating the sequences, in an embodiment the third sequence is separated from the first sequence by up to two amino acid residues. In another embodiment the third sequence is separated from the fourth sequence by up to three amino acid residues.

In an embodiment of the above polypeptide the first sequence is Pro-Arg-X6-Pro-His-Pro-Arg-X1 (SEQ ID NO: 19), wherein X6 is an amino acid residue. In an embodiment X6 is Pro. In another embodiment X6 is Thr.

In an embodiment, the fourth sequence comprises at least 13 amino acid residues. In a specific embodiment, at least one of every three consecutive amino acid residues is Pro. In an embodiment the fourth sequence comprises Pro-Arg-Pro.

In a specific embodiment the fourth sequence is selected from the group consisting of:
Ser-Gln-Pro-Arg-Pro-Gln-Pro (SEQ ID NO: 20),
Gln-Val-Pro-Ile-Arg-Pro-Ser-Gln-Pro-Arg-Pro-Gln-Pro (SEQ ID NO: 21), and
Ser-Arg-Pro-Ser-Pro-Gln-Val-Pro-Ile-Arg-Pro-Ser-Gln-Pro-Arg-Pro-Gln-Pro (SEQ ID NO: 22).

This invention further provides nucleic acid encoding the purified antibacterial polypeptide. The nucleic acid molecule can be either DNA or RNA, single stranded or double stranded. The single stranded molecule can be either the top (coding) or bottom (noncoding) strand. The single stranded nucleic acid molecule is useful as a

probe. In a preferred embodiment this invention provides for a plasmid capable of expressing the polypeptide. The plasmid contains transcriptional and translational control sequences known to those of skill in the art.

This invention provides a purified antibody capable of binding to the polypeptide described herein. In an embodiment the antibody is a rabbit antibody. In one embodiment the antibody is a polyclonal antibody. In another embodiment the antibody is a monoclonal antibody.

This invention further provides a method for obtaining the purified antibody capable of binding to the polypeptide described herein comprising: coupling an antigen selected from the group consisting of honeybee apidaecin and the polypeptide to a carrier protein; immunizing a mammal with the coupled antigen; and isolating the antibody from the mammal, thereby obtaining the purified antibody.

The coupling of an antigen which is poorly immunogenic to a carrier protein is known to those of skill in the art. Various carrier proteins are known to those of skill in the art. In an embodiment of the above method for obtaining the purified antibody capable of binding to the polypeptide described herein, the carrier protein is tuberculin purified protein derivative. In an embodiment the immunizing is immunizing by injecting. In a preferred embodiment the mammal is a rabbit.

This invention provides a method for determining the presence of the polypeptide in a sample comprising: incubating the sample with the antibody described above, and detecting an antibody-antigen complex, thereby determining the presence of the polypeptide in the sample.

In the above method, the step of detecting the antibody-antigen complex can be performed in a number of ways known to one of skill in the art. In a preferred embodiment, the detecting comprises detecting by enzyme-linked immunoassay. In another embodiment, the detecting comprises radioimmunoassay.

This invention provides a method for inhibiting growth of a bacterium comprising administering to the bacterium a growth inhibiting effective concentration of the polypeptide described herein.

This invention provides a method for determining the presence of the polypeptide in a sample comprising: incubating the sample with the antibody described above, and detecting an antibody-antigen complex, thereby determining the presence of the polypeptide in the sample.

In the above method, the step of detecting the antibody-antigen complex can be performed in a number of ways known to one of skill in the art. In a preferred embodiment, the detecting comprises detecting by enzyme-linked immunoassay. In another embodiment, the detecting comprises radioimmunoassay.

This invention provides a method for inhibiting growth of a bacterium comprising administering to the bacterium a growth inhibiting effective concentration of the polypeptide described herein.

This invention provides a pharmaceutical composition comprising an antibacterial effective amount of the polypeptide described herein and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are known to those with skill in the art. Examples include buffered saline solution and starch. In specific

embodiments the pharmaceutical composition is a liquid, a cream, or a solid such as a tablet.

This invention provides a method for treating a subject infected with a bacterium comprising administering to the subject an antibacterial effective amount of a polypeptide described above, thereby treating the subject. In an embodiment, the subject is a mammalian subject. In a more specific embodiment the subject is a human subject. The polypeptide is administered according to techniques known to those of skill in the art, including orally, parenterally, intraperitoneally, by intramuscular injection, by intravenous injection, or topically.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

EXPERIMENTAL DETAILS

First Series of Experiments

INTRODUCTION: Isolation and structural characterization of 13 novel, naturally occurring apidaecin-type peptides, and functional analysis (antibacterial spectra against medically relevant strains) of 17 members of this class of antibiotics are reported. Evolutionary 'conserved' and 'variable' regions in the apidaecin structure are delineated. Whereas it is speculated that conserved structures are responsible for general antibacterial capacity, it is clearly demonstrated that the natural diversity of the variable regions confers specificity to the antibacterial spectrum of each analog. Ability of certain homologs to overcome bacterial resistance against

related peptides also resides in variable region biodiversity.

EXPERIMENTAL PROCEDURES

Immuno-induction of insects: Hymenopteran insects used for immuno-induction are listed in table 1 and were obtained from (collected at) the following sources (locations): *N. autumnalis* from Dr. Michael R. Wagner (Forest Pest Management, Northern Arizona University, Flagstaff, AZ), *C. desantisi* and *G. legneri* from Dr. Richard Tassan (Division of Biological Control, U. of California, Berkeley, CA), *C. disparis* and *B. intermedia* from Dr. Paul W. Schaefer (Beneficial Insects Introduction Research, USDA-ARS North Atlantic Region, Newark, DE), *S. speciosus* from Central Park (NYC, NY), *V. maculifrons* from underground nest (West Milford, NJ), *V. maculata* from tree nest (Middletown, NJ), *P. germanica* from underground nest (Erpe Mere, Belgium), *C. pennsylvanicus* from Carolina Biological Supply Company (Burlington, NC), *A. mellifera* from Mr. Robert Cornetto (West Milford, NJ), *B. terrestris* from Prof. Frans Jacobs (Insect Research Center, Ghent State University, Belgium). Insects (1 to 10, depending on availability) were injected with 1-5 x 10^4 (depending on insect size) viable E. coli cells (ATCC 11775) suspended in 1 μl phosphate-buffered saline (PBS[1]: 0.15M, pH 7.2) A glass capillary (narrowed in the flame) was used for this purpose and alternatively, for the smallest insects such as the parasitic wasps, infections were carried out by clipping one wing with micro scissors or puncturing a leg with the tip of a hypodermic needle dipped for at least 30 seconds in a suspension of the same *E. coli* cells ($10^6/\mu$l). Insects were always sedated using CO_2 during manipulations. One day after infection, the insects were bled by puncturing the abdomen with a glass capillary. The collected hemolymph (0.5-2μl) was pooled in ice-

cooled tubes containing 100µl of 2%TFA to prevent proteolytic degradation of the immuno-induced peptides and to precipitate proteins. Owing to their small size, total extracts were prepared from *C .desantisi* and *G. legneri* by homogenizing them in 2%TFA. The precipitate was spun down and the clear supernatant was frozen at -80°C.

Table I. Insects screened for presence of apidaecin-type peptides:

CLASS: Hexapoda (insecta)
ORDER: Hymenoptera

SUBORDER	SUPERFAMILY	FAMILY	SPECIES
Symphyta	Tenthredinoida	Diprionidae	*Neodiprion autumnalis* (conifer sawflies)
Apocrita	Ichneumonoidea	Ichneumonidae	*Coccygomimus disparis* (parasitic wasps)
	Chalcidoidea	Encyrtidae	*Copedosoma desantisi* (chalcids)
		Chalcididae	*Brachymeria intermedia* (chalcids)
	Bethyloidea	Bethylidae	*Goniozus legneri* (parasitic wasps)
	Formicidae	(family not in superfamily)	*Camponotos pennsylvanicus* (carpenter ants)
	Vespoidea	Vespidae	*Vespula maculifrons* (yellow jackets)
			Vespula maculata (baldfaced hornets)
			Paravespula germania (german wasps)
	Sphecoidea	Sphecidae	*Sphecius speciosus* (cicada killers)
	Apoidea	Apidea	*Apis mellifera* (honeybees)
			Bombus terrestris (bumble bees)

Classification from Borror, De Long and Triplehorn (1981) An Introduction to the Study of Insects, 5th edition (W.B. Saunders, Philadelphia).

Reversed-phase high performance liquid chromatography: Depending on availability of "immune" lymph the primary fractionation by RP-HPLC was done on a 4.6 or 2.1x250 mm Vydac C4 (214TP54 or 214TP52) column from the Separation Group (Hesperia, CA) or on a 1x100mm Inertsil 100GL-1-ODS-I10/5 C18 column from SGE (Ringwood, Australia). Standard bore (4.6mm) columns were operated as described (1) using an AB 150A system (Applied Biosystems, Foster City, CA). Solvent A was 0.1% TFA (pH 2) and solvent B: 70% acetonitrile (MeCN) in A. Fractions were eluted at 1ml/min, with a three-step linear gradient: 0-50%B/50min, 50-70%B/10min, 70-100%B/8min (68min total time), UV detection was done at 214 nm. A modular LC system was used for chromatography on 2.1 mm columns. Basic components of the system were an AB 140B syringe pump and an AB 1000S diode array detector. Full details about system assembly, plumbing, operational parameters and solvents have been described (29). Microbore columns (1 mm) were operated in a similar instrument, except that an AB model 783 variable wavelength detector, fitted with a LC-packings (San Francisco, CA) Kratos-compatible capillary flow cell which was directly connected to the column outlet was used; gradient slope was 1% B/min at a flow of 30µl/min. Fractions were collected using a Pharmacia (Piscataway, NJ) Frac 100 automated instrument (for the 4.6 mm column) or by hand (for the 2.1 and 1mm columns) and put on ice; aliquots were removed at this point for ELISA, MS-analysis and analytical LC (on a smaller column). Repurifications were done on a variety of columns (different manufacturer, carbon chain length and carbon load, than the one for the primary run): Vydac C18, Vydac diphenyl, Aquapore RP300 (C8). Aside from the real-time stripchart recordings, chromatograms were also obtained on a PE Nelson (Cupertino, CA) data system using the 2700 Turbochrom (version 3) software.

Anti-apidaecin polyclonal antisera: For adequate

immunization of rabbits with apidaecin, the peptide had to be coupled to a tuberculin purified protein derivative (PPD) using a Cambridge Research Biochemicals (Cambridge, UK) immunization kit. Conjugation was done via the N-terminal amino group using glutaraldehyde and following the manufacturers protocol, except for some small modifications. Briefly, to the reaction vial, containing 75µl PPD solution (10mg/ml in 0.1M sodium hydrogen carbonate buffer pH 8.4), apidaecin (200µg dissolved in 75µl of the same buffer) was added together with 5µl glutaraldehyde (10% in buffer). The mixture was agitated and kept on room temperature for 18h, five fold diluted with PBS and then dialyzed against PBS. The dialyzed solution (1ml) was used for immunization of a rabbit, pre-vaccinated (with live *Bacillus Calmette-Guerin*) three weeks before injection, following the manufacturers schedule. In this way, anti-apidaecin antiserum was obtained for use in further studies. By itself, apidaecin is a very poor antigen.

Enzymé-linked immuno assays (ELISA): Aliquots from RP-HPLC column fractions (20/500µl for 4.6 mm columns; 4/75µl for 2.1mm columns and 2/25µl for 1 mm columns) or samples in MilliQ water (Millipore) were tested for the presence of apidaecin-like molecules by alkaline phosphatase based ELISA using anti-apidaecin (bee Hb Ia) polyclonal antiserum as the primary antibody. Tests were carried out essentially as described (30). Briefly, aliquots (see above) were mixed with 80µl coat buffer (PBS pH=7.4 containing 6% saccharose and 1% PEG6000) in the wells of a 96-well micro titer plate (Costar EIA/RIA, Cambridge, MA) and incubated overnight at 4°C. The coat solution was discarded and the plates were blocked with 200µl of a BSA solution (0.1%) in PBS for 1h/RT, followed by two washes with PBS. Rabbit anti-apidaecin (bee) polyclonal antiserum (diluted 1/1000 in PBS/BSA) was then added and incubated for 1hr/RT; plates were then

washed five times with 0.1% Tween 80 in PBS. Subsequently, alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma; diluted 1/1000 in PBS, containing 10mg/ml BSA) was added, plates were incubated for 1h/RT and then washed 5 times with the Tween/PBS solution. Enzyme substrate (1mg/μl p-nitrophenyl phosphate in 10% diethanolamine, containing 0.5 mM $MgCl_2$ and 0.02% NaN_3, pH9.2) was then added for 30 min/RT and the reaction terminated by addition of 50μl 0.1M EDTA. Positives were scored by monitoring yellow color development though visual inspection and/or by measuring (at 405nm) in a Titertek Multiscan microplate reader. A dilution series of synthetic bee apidaecin (1, 0.1, 0.01, 0.001 μg/ml) was used as a control.

Mass Spectrometry (MS): HPLC column fractions, pure peaks and synthetic peptides were subjected to matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry (MS) analysis using a Vestec (Houston, TX) LaserTec instrument with a 337 nm output nitrogen laser and a 1.2m linear flight tube, as described (31). In general, 0.5μl sample (in 20% MeCN containing 0.1% TFA) was mixed with 1μl matrix (sinapinic acid or alpha-cyano-4-hydroxy cinnamic acid (ACCA)) solution, applied to the stainless steel probe tip and air dried before analysis. A 25 kV ion acceleration and 3 kV multiplier voltage were used. Typically, up to eight analyses were necessary for each data point, with varying matrices, sample concentrations and laser power. Laser power was varied between experiments as judged from optimal deflections of specific maxima, using a Tektronix (Beaverton, OR) TDA 520 digitizing scope. Small amounts (1 picomole, 100 and 10 femtomoles) of synthetic bee apidaecin Hb Ib (average isotopic mass MH^+= 2109.43) were added as an external control and to optimally calibrate the instrument. The nitrogen laser emits pulses of 400 microjoule at 337 nm and is therefore a class IIIb laser product. All safety

precautions were taken as set forth in booklet ANS Z136.1 of the Laser Institute of America.

Peptide sequencing: Purified apidaecin-like peptides were sequenced with the aid of an Applied Biosystems model 477A automated sequenator, operated according to the principles outlined by Hewick et al (32). Stepwise liberated PTH-amino acids were identified using an "on-line" 120A HPLC system equipped with a PTH C18 (2.1x220 mm; 5 micron particle size) column (AB). The standard AB method was optimized for sub-picomole PTH analysis as described (33,34). It is well known that proline residues are cleaved rather slowly with concomitant developing lag in the sequencing cycles. Because apidaecins are very Pro-rich, a special sequencing cycle with double TFA-cleavage time (from 350sec to 700sec) was used. Extremely valuable information for interpretation of sequencing experiments came from the preceding mass-analyses; this helped in deconvoluting the signals for certain cycles (31). In the end, of course, the theoretical mass of the peptide (from sequencing results) must match experimental mass (from MS).

Chemical peptide synthesis: Chemical synthesis of apidaecin-type peptides and pig cecropin P1was performed with an automated peptide synthesizer, model 430A (Applied Biosystems). 9-Fluoroenylmethoxycarbonyl (Fmoc)-N-protected, L-configuration amino acids were coupled sequentially to 4-hydroxymethylphenoxyacetic acid (HMP). Side chain protecting groups were: Asn, Gln and His (trityl), Arg (4-methoxy-2,3,6-trimethylbenzenesulfonyl) and Tyr (t-butyl). The loading of the starting resin was 0.25 mmole (0.284g HMP). The standard Applied Biosystems (AB) synthesis protocol 'FastMoc' was used, except that the first amino acid was triply coupled and the remaining free sites were blocked using acetic anhydride. All reagents and solvents were

from AB. After completion of synthesis and removal of N-Fmoc group, 500mg of the neutralized dried resin was cleaved for 4 hours in a 10ml PolyPrep chromatography column (Biorad) with 10ml of 82.5% TFA/ 5% phenol/ 5% thioanisole/ 5% water (V/V). After elution of the solution in a 50 ml conical vial, peptides were pelleted by precipitation in 25ml ice cold tert-methyl butyl ether (3 times) and centrifugation, followed by solubilization in 20% acetic acid at a concentration of 25mg/ml. The next step was to preparatively purify all peptides on a 2x25cm Vydac C4 column, using the earlier described acetonitrile/TFA based solvent system, operated at a flow of 12ml/min and a gradient slope of 1%B/min. The HPLC system was comprised of two model HPX pumps (Rainin Instruments, Woburn, MA) and an AB model 1000S diode array detector equipped with a preparative flow cell. Quality control of purified material was done by analytical HPLC and mass spectrometry before use in antibacterial tests.

Quantitation of peptides: Stock solutions of all apidaecin-type peptides and cecropin P1, in highly purified water (MilliQ system, Millipore) or dilute acid (0.1-1% TFA), were quantitated by amino acid analysis. An automated amino acid analyzer with vapor-phase hydrolysis, AB model 420, was used for this purpose. Stocks were stored at -70°C and periodically requantitated before dilution and use in activity tests. A quick way to assess approximate levels of apidaecin in solutions (pure or crude) is by analytical HPLC, as a measure of peak surface ($6x10^5 \mu V.sec$ corresponds to $1 \mu g$ peptide). The calibration curve hereby used was obtained from analyzing a dilution series of synthetic products. The calibration is linear in the 0.05 to 50 $\mu g/ml$ range with a maximal error margin of 10% (35).

Bacterial strains: All bacterial strains (except three)

used in this study were obtained from the American Type Culture Collection (ATCC, Rockville, MD); reasons for selection are discussed in the text. Grouping, according to Bergey's Manual of Determinative Bacteriology, ninth edition (Williams & Wilkins, Baltimore, MD), and strain numbers are as follows: Group 2: *Campylobacter jejuni* ATCC 33560, *Helicobacter pylori* ATCC 43504; Group 4A: *Acinetobacter calcoaceticus* ATCC 49137, *Agrobacterium tumefaciens* ATCC 15955, *Bordetella pertussis* ATCC 9340, *Flavobacterium meningosepticum* ATCC 13253, *Francisella tularensis* ATCC 6223, *Legionella pneumophila* ATCC 33152, *Neisseria gonorrhoeae* ATCC 19424, *N. meningiditis* ATCC 13077, *Pseudomonas aeruginosa* ATCC 10145, *P. syringae* NCPPB 1106, *Rhizobium meliloti* ATCC 10310, *Xanthomonas maltophilia* ATCC 12714; Group 4B: *Bacteriodes melaninogenicus* ATCC 25845; Group 5.1 ('Enterobacteriaceae'): *Citrobacter freundii* ATCC 8090, *Escherichia coli* ATCC 25922 (clinical isolate) and 11775, *E.coli* 11775 Apid^R (apidaecin-resistant strain derived from strain ATCC11775, see text), *E. coli* K514 (common strain from molecular cloning laboratory), *Enterobacter cloacae* ATCC 529, *Erwinia* amylovora ATCC 15580, *Klebsiella pneumoniae* ATCC 13883, *Morganella morganii* ATCC 25830, *Proteus mirabilis* ATCC 25933, *Salmonella typhimurium* ATCC 14028, *S. typhi* ATCC 6539, *Shigella dysenteriae* ATCC 13313, *Yersinia enterocolitica* ATCC 9610; Group 5.3: *Haemophilus ducreyi* ATCC 33940, *H. influenzae* ATCC 19418; Group 5.4: *Gardnerella vaginalis* ATCC 14018. All strains were handled under conditions and in a laboratory environment in compliance with Biosafety Level 2 for Infections Agents as set forth in HHS Publication No. (NIM) 88-8395 ("Biosafety in microbiological and biomedical laboratories"). At the conclusion of all experiments, the area was decontaminated and microorganisms destroyed by autoclaving.

Inhibition zone assay: Aliquots of all synthetic, purified, apidaecin-type peptide stocks were diluted (to 2.5nmol/μl final concentration) in MilliQ water. Aliquots of 20 μl and controls (20μl MQ water) were then applied in 3mm diameter wells on agar plates seeded with log phase bacteria. Plates were incubated at 28°C (plant associated bacteria and *E.cloacae, H. ducreyi, A. calcoaceticus & Y. enterocolitica)* or 37°C (others) and inspected for inhibition zone development after 24 or 48h; inhibition zones were then measured. In general, bacteria were grown on BHI (brain heart infusion) medium (Difco 0003), except for *Agrobacterium, Erwinia, Pseudomonas syringae, Acinetobacter* and *Morganella* which were grown on nutrient agar, *Campylobacter, Francisella, Gardnerella, Bacteriodes, Bordetella* and *Haemophilus ducreyi* on chocolate agar (Remel 01-300 plates), *Legionella* on cye agar (Remel 01-342 plates), *Haemophilus influenzae* and *Helicobacter* on GC medium (Difco 0289) with 2% hemoglobin powder (BBL 11871) and *Rhizobium* on tryptic soy agar. All tests were done under aerobic conditions, except for *Campylobacter* and *Helicobacter* which were grown under micro-aerophilic conditions ('campypak') and *Neisseria , Legionella* and *Haemophilus ducreyi* under 5-10% CO_2 ('CO_2-pak').

Minimal Inhibitory Concentrations: Minimal inhibitory concentrations (MIC's) of all apidaecin-type peptides against selected bacterial strains were determined in flat-bottomed 96-well microtiter plates (Microtest III Tissue culture plate, Falcon); 10μl aliquots of serial dilutions of peptides were added to 70 μl deionized water (MQ) and the mixture was in
oculated with 20 μl of a bacterial suspension containing $3x10^5$ (or less) viable cells in the appropriate growth medium (typically the same as for agar plate tests but minus the agar). Final peptide concentrations were 0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 5, 10 and 20 μg/ml.

The MIC values (a-b) express the highest peptide concentration at which cells were able to grow (a) and the lowest concentration at which no growth was observed (b), both after incubation at 28°/37°C for at least 48h.

RESULTS

Purification of novel Apidaecin-type peptides. Structural constraints on antibacterial capacity of apidaecin through sequence comparison of homologous peptides, isolated from different insects were investigated. Previous, extensive studies have failed to detect the presence of apidaecin-type peptides in any insects outside the Hymenoptera order. Thus, twelve hymenopteran insects (listed in table 1) were chosen for analysis. They have been selected to represent both suborders (*Symphyta* and *Apocrita*) and seven out of the thirteen superfamilies (including all families not placed in superfamilies) that make up the largest (*Apocrita*) suborder (according to the classification in Ref. 27). Some of the species belong to the same super-families, or the same families even: honeybees and bumblebees (*Apidae* family); hornets, yellow jackets and german wasps (*Vespidae* family); one representative of the *Encyrtidae* family, one representative of the *Chalcididae* family (both members of the *Chalcidoidea* superfamily). It was felt that this should enable a determination of close and distant molecular evolutionary relationships, casting more light on the degree of conservation (and functional significance) of particular residues or stretches of sequence in the apidaecin molecule. Because the *Symphyta* suborder is very distinct from all other hymenopteran insects, there was a reasonable risk that they did not contain apidaecin-like peptides. Therefore, only one member of this suborder was chosen for study.

Immuno-induction was carried out on all selected insects

(table 1) and 'immune' lymph was harvested or total extracts were prepared. Acid stable molecules in 'crude' hemolymph or total extracts were fractionated by HPLC, and column fractions were screened for apidaecin homologs using enzyme-linked immuno assay (ELISA) with anti-apidaecin (bee Ib) antiserum. Positive peptide peaks were first checked by MALDI-TOF MS for the presence of molecules with M_r in the 1,500 to 2,500 dalton range and then further purified to apparent homogeneity. To make sure that any 'apidaecin-like' peptides that did not cross-react with the antiserum and/or fell outside the expected molecular size brackets would not be overlooked, antibacterial testing against *E. coli* ATCC 11775 and *Bacillus megaterium* QMB1551 and *Arthrobacter sp.* NRRLB 3724 were carried out. Several additional antibacterial 'factors' were thus isolated (e.g. from chalcids and carpenter ants) and subsequently shown, by limited structural analysis, to be devoid of PP or PRP sequences (data not shown) that are characteristic for the Pro-Arg-rich family of peptide antibiotics. Due to insufficient source material, antibacterial testing was omitted on fractions derived from *C. desantisi* and *G. legneri*.

Following intense scrutiny, it appeared that not all hymenopteran insects secreted detectable levels of apidaecin-type peptides into their hemolymph in response to *E. coli* infections. They did however, produce other 'response-factors', some of which had outspoken antibacterial activities (2). In contrast, the cicada-killer wasp (*Sphecious speciosus*) produced a massive quantity of apidaecin (two different isoforms) but no significant levels of any other peptides.

Covalent structures. From the earliest isolation and sequencing experiments, the presence of three apidaecin isoforms in honeybees (24) was known; the structures are shown in figure 1. While isoform Ib is clearly

predominant in 'immune' lymph (90%), antibacterial spectra and specific activities are the same for all three peptides (24). More recent analysis of apidaecin cDNA clones indicated that the various peptides are generated by processing of single polyprotein precursors (35). Analysis of all putative isoforms, observed in the open reading frames, indicated the presence of a fourth species, apidaecin III (with Pro replaced by Ser at position 9; see figure 1), that had never been found in lymph.

Covalent structures of all newly isolated apidaecin-type peptides were studied by a combination of chemical micro-sequencing and mass spectrometry. Sequences are listed, in aligned format, in figure 1. Also shown in that table are theoretical (average isotopic mass) molecular weight values [MH^+], calculated from the proposed sequences using Procomp version 1.2 software (kindly provided by Dr. P.C. Andrews, Michigan U., Ann Arbor, MI), and the experimentally obtained *m/z* values. Only in a single case is the difference between theoretical and experimental mass >1.0 dalton (>0.05% error), namely for hornet peptide Ho+, where a discrepancy of 1.67 dalton was observed; this is still within limits of experimental error. Thus, chances that any of these peptides carry post-translational modifying moieties are virtually non-existing.

As is evident from the results shown in figure 1, micro-heterogeneities, in the form of ragged ends (likely due to incomplete processing or unfavorable exoproteolysis) and various isoforms, occur within certain insect species (honeybees, bumble bee, cicada killer, *Coccygomimus*). They were not easily separated by RP-HPLC, due in part to peak-broadening caused by racemization of proline bonds. Edman-sequencing data were further confounded by the abundance of proline residues, causing additional lag.

The accurate mass measurements allowed deconvolution of the mixed chemical sequencing results. A detailed account of these Edman-chemical / MALDI-TOF MS approaches to micro peptide sequencing has been given elsewhere (31).

From the proposed alignment in figure 1, it could be concluded that certain parts of the apidaecin-type peptides are indeed evolutionary conserved, notably a carboxy-terminally located stretch of eight amino acids (PRPPHPRL) (SEQ ID NO:31), a R/K-P dipeptide (residues 4-5 in the Hb Ib sequence numbering) and a proline at position 9 (except in Hb III). Intervening and amino-terminal regions seem to be 'variable'. Not unexpectedly, these variations are rather subtle (single or double amino acid substitutions) between peptides from closely related insects (e.g. honeybees / bumble bees; wasps / hornets / yellow jackets) and more pronounced between, for instance, bees and distantly related parasitic wasps from the *Ichneumonoidae* superfamily. The only surprise was that, while peptides from two members of the genus *Vespula* (yellow jackets and hornets) were slightly different (by one residue), one of them (from yellow jackets) was identical to the major apidaecin-form isolated from a different genus (*Paravespula*; german wasp); this is mildly puzzling in that it seemingly violates the generally accepted correlation between taxonomic classification and molecular evolution (36).

Antibacterial spectra. To examine possible effects of the observed sequence differences on antibacterial capacity and spectrum, testing against thirty two selected bacterial strains was initiated (see 'Experimental Procedures') using agar plate growth inhibition zone assays. These bacteria have been specifically chosen to bring together a relevant selection of non-virulent strains. Relevant, because they

are representatives of bacterial species typically used as test-strains for evaluation of novel antibiotics (e.g. *E. coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Citrobacter freundii*, *Proteus mirabilis*, *Morganella morganii*, *Acinetobacter calcoaceticus*, *Yersinia enterocolitica*, *Haemophilus influenzae*, *Salmonella*, *Neisseria gonorrhoeae*, *N. meningiditis*, *Xanthomonas maltophilia* and *Pseudomonas aeruginosa*) (37,38), and of such selected disease-causing microbes as *Bordetella pertussis* (whooping cough), *Francisella tularensis* (tularemia), *Legionella pneumophila* (form of pneumonia), *Gardnerella vaginalis* (ulcers), *Campylobacter jejuni* (enteric pathogen; estimated 2 million infections per year in the U.S.) and *Helicobacter pylori* (gastric ulcers) (39,40). As the *Salmonella typhimurium* strain, strain ATCC14028 was selected because it has been used extensively for genetic studies of virulence, including resistance against defensins (41). Four plant-associated bacteria were also included (as positive controls so to speak, since honeybee-derived antimicrobials are very active against them). *E. coli* strain 11775Apid^R is a mutant derived from strain ATCC 11775 and is resistant to approximately 500-fold higher apidaecin (type Hb Ib) concentrations than the parental strain (MIC value against 10^7 cells/ml of 50 μg/ml compared to 0.1 μg/ml) (25). Interestingly, mutant and parental strain are equally susceptible to abaecin, a different Pro-rich 'immune' peptide from honeybees (42)

Due to limited availability, these studies started with rather small numbers of insects (e.g. 20 hornets, 4 cicada-killer wasps, 4 small parasitic wasps). As a result, only low picomolar quantities of purified peptides were available, largely insufficient to carry out antibacterial testing on the proposed scale. All known, naturally occuring apidaecin-type peptides (listed in figure 1) were therefore chemically synthesized,

purified (50 mg each), quality controlled (by MS), and stock solutions made and quantitated (by amino acid composition analysis). Peptides Hb Ia and II were not included in further studies as they have been shown previously to be functionally similar to Hb Ib (24,43). Peptides Ho- and Cd2- have never been observed in nature but were included here to study the possible functional significance of the N-terminal GKP sequence. Cecropin P1 (synthetic product based on the published sequence (44) of a peptide isolated from pig intestine) was included as a representative of mammalian peptide antibiotics. Selection of this antibacterial peptide as a 'control' was based on the assumption that, because of the tissue and specific anatomical site from which it was isolated, activities against enteric bacteria and against certain pathogens that enter the body through the gastro-intestinal tract were to be expected.

Results from antibacterial testing (in triplicate) of sixteen apidaecin-type peptides and cecropin P1 against the thirty two aforementioned bacterial strains are summarized in table 2. A number of observations can be readily made. 1) Artificial peptide Hb III, based on a gene sequence, and the only peptide carrying a substitution of conserved Pro(9), is essentially inactive against nearly all strains tested. 2) Several apidaecin-type peptides are clearly active against mutant *E. coli* strain 1175Apid^R, whereas others (including the 'original' honeybee peptide Ib) are most definitely not. 3) Additional functional variability, in terms of antibacterial spectra and specific activities, exists among apidaecin-type peptides, to the extent that apidaecin-analog-based antibiograms against certain bacteria are nearly 'mirror image' of one another (e.g. *Yersinia enterocolitica* compared to *Campylobacter jejuni*). 4) As a group, *Coccygomimus*-derived apidaecin-type peptides are somewhat less active against

Enterobacteriaceae than all the other ones, but have moderately to significantly better activities against *Campylobacter jejuni*, *Legionella pneumophila* and *Haemophilus influenzae*. 5) All apidaecin-type peptides are completely inactive against several of the test strains; cecropin P1 has measurable activity against some of those resistant strains (e.g. *Haemophilus ducreyi*, *Xanthomonas maltophilia* and *Bacteriodes melaninogenicus*) but not against all (e.g. *Neisseria* strains, *Proteus*, *Gardnerella* and *Helicobacter pylori*). 6) Finally, and not unexpectedly, Apidaecin-type peptides have extraordinary activities against plant-associated bacteria (*Erwinia*, *Agrobacterium tumefaciens*, *Rhizobium meliloti* and *Pseudomonas syringae*); even defective peptide Hb III showed considerable activity whereas the otherwise very potent cecropin P1 did not (see *Rhizobium* and *P. syringae*, for instance).

Careful scrutiny of the apidaecin-type sequences (figure 1) and of the antibiograms in table 2 indicates that contrasting antibacterial spectra/specificities can be correlated, in several cases, with very subtle sequence differences. Table 3 contains illustrative examples of these fascinating structure/function correlations. Peptides Cd3-,2-,1- differ by just one (S to N), or only two (K/K to R/Q) or three (S/K/K to N/R/Q) amino acids; the rest of the sequences are identical (see figure 1, table 3A). Thus, R/Q (at positions 4/10) confers strong antibacterial activity to apidaecins against *S. typhimurium* and *Yersinia enterocolitica* but incapacitates the same peptides as antibacterial agents against *Campylobacter jejuni* and *Legionella pneumophila*; K/K (at 4/10) totally reverses this specificity profile (table 4A). Peptide Hb Ib cosegregates with Cd-1 from Cd2-/3- by virtue of its specificity against the eight test strains listed in table 4. Interestingly, while substantially divergent in sequence from all three Cd-

peptides, Hb Ib contains the same characteristic N/R/Q (3/4/10) motif as Cd2-, leaning further support to the notion that R/Q (4/10) is a specificity determining motif. As for the difference between Cd2- and Cd3-, the S to N (at position 3) substitution increases the specific activity against *Erwinia amylovora*. Peptides Ho+ and Yj-S also differ by just one residue (G to N) (see figure 1; table 3B). While the 'G'-form has substantially higher specific activities against *Francisella tularensis* and *Morganella morganii* than the 'N'-form, a full reversal of this specificity against *Acinetobacter calcoaceticus* and *Erwinia amylovora* is seen; no functional differences between the two peptides were observed against several *E.coli* strains. Ho/Yj- is identical to the former two peptides but lacks the first three amino acids (figure 1 and 3B). This G/N-K-P truncation eliminates antibacterial activities against many, but not all, bacteria; activities against the *Morganella* strain and *E. coli* ATCC11775 were unaffected. What this actually means, is that the presence or absence of these three amino acids at the amino-terminus of some apidaecin-type peptides critically determines capacity to overcome antibacterial resistance of *E. coli* mutant strain '11775apid^R'. A similar effect of a GKP truncation was observed for peptide Cd2+ (termed Cd2- after truncation), with a decrease of antibacterial activity against *Campylobacter*, *Haemophilus* and both *Salmonella* strains but with no measurable effect on activities against all other sensitive bacteria tested (table 2).

Key to Table II. Antibacterial spectra of sixteen apidaecin-type peptides.

Listed are the results of agar plate inhibition zone assays. Bacterial strain identification and growth conditions are given in 'Experimental Procedures'. Peptides (listed in top row; see figure 1 for abbreviation legends) were applied in 3 mm wells, 50

nanomoles/well for all apidaecins and 5 nanomoles/well for cecropin P1 (CP1). Number of plus signs express the diameter of the inhibition zones and can be read using the following key: (+) 5-6 mm; (++) 7-9 mm; (+++) 10-14 mm; (++++) 15-19 mm; (+++++) 20 or more mm. (-) denotes that no inhibition was observed. Growth of *Helicobacter pylori*, *Flavobacterium meningosepticum*, *Neisseria gonorrhoeae*, *N. meningiditis*, *Gardnerella vaginalis*, *Proteus mirabilis*, *Bordetella pertussis*, *Xanthomonas maltophilia*, *Haemophilus ducreyi*, *Bacteriodes melaninogenicus* and *Pseudomonas aeruginosa* was not inhibited by any of the apidaecin-type peptides.

Key to Table III. Effects of subtle amino acid substitutions on antibacterial specificity of apidaecin-type peptides.

Listed is a small selection of the agar plate inhibition zone results from table 2; see table 2 for experimental conditions and scoring system. Specific amino acids present in a particular position in the peptide sequences (for numbering, see figure 1) are also listed. Peptides Cd3-, 2-, and 1- are identical except for the amino acid differences shown; HbIb differs by additional residues. Peptides Ho+ and Yj-S are also identical except for a G to N change in position 2a; (/) indicates the absence of amino acids.

Key to Table IV. Antibacterial actvities of apidaecin-type peptides.

The minimal inhibitory concentration (MIC) of sixteen apidaecin-type peptides and cecropin P1 to inhibit growth of some representative strains are expressed in μg/ml. For details see 'Experimental Procedures'. Inoculum (in CFU/ml) is listed for each bacterial strain; *E. coli* strains ATCC11775 and 11775apid^R were tested with two different inoculum sizes.

Table II/Panel A Antibacterial spectra of sixteen apidaecin-type peptides.

Bacteria	Hb Ib	Hb III	Bb-A	Bb+A
Enterobacteriaceae				
Escherichia coli (ATCC11775)	++++	–	++++	++++
Escherichia coli (11775 Apid R)	–	–	–	–
Escherichia coli (K514)	+++	–	+++	+++
Enterobacter cloacae	+++	–	+++	+++
Erwinia amylovora	++++	–	++++	++++
Klebsiella pneumoniae	+++	+	++	++
Morganella morganii	+	–	–	–
Salmonella typhimurium	+++	+	+++	+++
Salmonella typhi	+++	+	+++	+++
Shigella dysenteriae	++++	+	+++	++++
Yersinia enterocolitica	+++	++	+++	+++
Other Bacteria				
Campylobacter jejuni	–	–	–	–
Acinetobacter calcoaceticus	+	–	++	++
Agrobacterium tumefaciens	+++++	++++	++++	++++
Francisella tularensis	+++	+	+++	+++
Legionella pneumophila	–	–	+	++
Pseudomonas syringae	++++	++	++++	+++++
Rhizobium meliloti	+++++	+++++	+++++	++++
Haemophilus influenzae	+++	nt	++	+++

Table II/Panel B Antibacterial spectra of sixteen apidaecin-type peptides.

Bacteria	Ck P	Ck A	Ho -	Ho +
Enterobacteriaceae				
Escherichia coli (ATCC11775)	+++	+++	+++	+++
Escherichia coli (11775 Apid R)	–	–	–	+++
Escherichia coli (K514)	++	++	–	+++
Enterobacter cloacae	+++	++	+	+++
Erwinia amylovora	+++	+++	++	+++
Klebsiella pneumoniae	++	++	+	+++
Morganella morganii	–	–	++	++
Salmonella typhimurium	++++	+++	+	+++
Salmonella typhi	+++	+++	+	+++
Shigella dysenteriae	++++	+++	+	+++
Yersinia enterocolitica	+++	+++	++	++++
Other Bacteria				
Campylobacter jejuni	–	–	–	–
Acinetobacter calcoaceticus	–	–	–	+
Agrobacterium tumefaciens	++++	++++	–	++
Francisella tularensis	++	+	–	+++
Legionella pneumophila	–	–	–	–
Pseudomonas syringae	++++	+++	+++	+++
Rhizobium meliloti	+++++	++++	++++	++++
Haemophilus influenzae	++	++	–	+++

Table II/Panel C Antibacterial spectra of sixteen apidaecin-type peptides.

Bacteria	Yj +S	Yj -S	Cd 1+	Cd 1-
Enterobacteriaceae				
Escherichia coli (ATCC11775)	+++	+++	++	+++
Escherichia coli (11775 Apid R)	+++	+++	++	++
Escherichia coli (K514)	++	+++	++	+++
Enterobacter cloacae	++	++	+++	+++
Erwinia amylovora	+++	++++	++	++++
Klebsiella pneumoniae	++	++	++	++
Morganella morganii	–	–	++	++
Salmonella typhimurium	+++	+++	++	+++
Salmonella typhi	++	+++	+++	++
Shigella dysenteriae	++++	+++	++++	+++
Yersinia enterocolitica	–	++	–	++
Other Bacteria				
Campylobacter jejuni	–	–	+++	–
Acinetobacter calcoaceticus	+	++	++	++
Agrobacterium tumefaciens	–	++	++++	+++
Francisella tularensis	++	+	+++	++
Legionella pneumophila	–	–	++	–
Pseudomonas syringae	++++	++++	++++	++++
Rhizobium meliloti	nt	++++	++++	++++
Haemophilus influenzae	+++	++	++++	+++

Table II/Panel D Antibacterial spectra of sixteen apidaecin-type peptides.

Bacteria	Cd 2+	Cd 2-	Cd 3+	Cd 3-	CP-1 500p
Enterobacteriaceae					
Escherichia coli (ATCC11775)	+++	+++	++	++	–
Escherichia coli (i1775 Apid R)	+++	++	++	++	–
Escherichia coli (K514)	++	++	+	+	+
Enterobacter cloacae	+++	+++	+++	+++	++++
Erwinia amylovora	++	+++	+	+	nt
Klebsiella pneumoniae	++	++	++	++	+
Morganella morganii	++	++	++	++	–
Salmonella typhimurium	+++	+	++	++	++
Salmonella typhi	+++	+	++	–	+++++
Shigella dysenteriae	+++	++	+++	++	++++
Yersinia enterocolitica	–	–	–	–	+++++
Other Bacteria					
Campylobacter jejuni	+++++	+++	+++++	++++	+++
Acinetobacter calcoaceticus	+	+	+	+	++
Agrobacterium tumefaciens	+++	++++	+++	++	++++
Francisella tularensis	+++	+++	++++	+++	–
Legionella pneumophila	+++	++	++	++	+++++
Pseudomonas syringae	+++	+++	++	++	–
Rhizobium meliloti	++++	+++	+++	++++	+
Haemophilus influenzae	++++	++	++++	+++	++++

Table III/ Panel A1 Effects of subtle amino acid differences on antibacterial specificity of apidaecin-type peptides

Peptide	Amino acid @ position 3	4	5	Bacteria *E. coli K514*	*Erwinia amylovora*	*Pseudomonas syringae*	*Salmonella typhimurium*
Cd 3-	S	K	K	+	+	++	++
Cd 2-	N	K	K	++	+++	+++	+
Cd 1-	N	R	Q	+++	++++	++++	+++
Hb Ib	N	R	Q	+++	++++	++++	+++

Panel B1	2a	2b	2c	*E. coli 11775*	*E. coli ApidR*	*Agrobacterium tumefaciens*	*Haemophilus influenzae*
Ho +	G	K	P	+++	+++	++	+++
Yj-S	N	K	P	+++	+++	++	++
Ho -	/	/	/	+++	–	–	–

Table III/ Panel A2 Effects of subtle amino acid differences on antibacterial specificity of apidaecin-type peptides

Peptide	Amino acid @ position 3	4	5	Bacteria *Salmonella typhi*	*Yersinia enterocolitica*	*Campylobacter jejuni*	*Legionella pneumophila*
Cd 3-	S	K	K	–	–	++++	++
Cd 2-	N	K	K	+	–	+++	++
Cd 1-	N	R	Q	++	++	–	–
Hb Ib	N	R	Q	+++	+++	–	–

Panel B2	2a	2b	2c	*Francisella tularensis*	*Morganella morganii*	*Acinetobacter calcoaceticus*	*Erwinia amylovora*
Ho +	G	K	P	+++	++	+	+++
Yj-S	N	K	P	+	–	++	++++
Ho -	/	/	/	–	++	–	++

Table IV/Panel A Antibacterial activities of apidaecin-type peptides

Peptide	*E. coli* 11775 (1200 CFU/ml)	*E. coli* Apid^r (1000 CFU/ml)	*E. coli* 23802 (5000 CFU/ml)	*E. coli* 25922 (1100 CFU/ml)	*E. coli* 11775 ($16x10^6$CFU/ml)
Hb Ib	<0.05	20-40	10-20	0.5-1	1-5
Hb III	0.1-0.5	>40	>40	20-40	20-40
Bb - A	0.1-0.5	>40	10-20	1-5	1-5
Bb + A	0.05-0.1	>40	10-20	0.5-1	0.5-1
Ck P	0.1-0.5	>40	20-40	0.5-1	1-5
Ck A	0.1-0.5	>40	>40	1-5	1-5
Ho-	0.5-1	>40	>40	10-20	10-20
Ho+	0.1-0.5	1-5	0.5-1	0.1-0.5	0.1-0.5
Yj+S	0.1-0.5	1-5	0.5-1	0.1-0.5	0.1-0.5
Yj-S	0.1-0.5	5-10	1-5	0.1-0.5	0.1-0.5
Cd 1+	10-20	10-20	10-20	10-20	>20
Cd 1-	0.5-1	>40	5-10	0.5-1	0.5-1
Cd 2+	0.1-0.5	0.5-1	0.1-0.5	0.1-0.5	0.1-0.5
Cd 2-	0.5-1	5-10	10-20	0.5-1	0.5-1
Cd 3+	0.1-0.5	0.5-1	0.1-0.5	0.1-0.5	0.1-0.5
Cd 3-	0.1-0.5	5-10	5-10	0.5-1	0.5-1
CP-1	0.2-1	0.2-1	0.2-1	0.2-1	0.2-1

The minimal inhibitory concentration (MIC) of sixteen apidaecin-type peptides and cecropin P1 to inhibit growth of some representative strains are expressed in μg/ml. For details see 'Experimental Procedures.' Inoculum (in CFU/ml) is listed for each bacterial strain; *E. coli* strains ATCC11775 and 11775Apid^r were tested with two different inoculum sizes.

Table IV/Panel B **Antibacterial activities of apidaecin-type peptides**

Peptide	*E. coli* Apid^r (12x10^{6}CFU/ml)	*S.typhimurium* (8x10^{6}CFU/ml)	*A.calcoaceticus* (16x10^6 CFU/ml)	*M.morganii* (400 CFU/ml)	*Y.enterocolitica* (5000 CFU/ml)
Hb Ib	>40	1-5	10-20	1-5	1-5
Hb III	>40	>20	>20	>40	>40
Bb - A	>40	0.5-1	>20	>40	1-5
Bb + A	>40	0.5-1	2-10	20-40	5-10
Ck P	>40	1-5	>20	>40	1-5
Ck A	>40	1-5	>20	20-40	1-5
Ho-	>40	5-10	>20	10-20	10-20
Ho+	5-10	0.5-1	2-10	1-5	1-5
Yj+S	10-20	0.1-0.5	2-10	0.5-1	1-5
Yj-S	5-10	0.1-0.5	2-10	1-5	1-5
Cd 1+	10-20	10-20	>20	10-20	20-40
Cd 1-	20-40	0.5-1	>20	20-40	5-10
Cd 2+	0.5-1.5	0.1-0.5	10-20	1-5	10-20
Cd 2-	10-20	1-5	10-20	1-5	>40
Cd 3+	0.5-1.5	0.1-0.5	10-20	1-5	10-20
Cd 3-	5-10	1-5	10-20	1-5	20-40
CP-1	0.2-1	1-2	2-10	0.2-1	0.2-1

Minimal inhibitory concentrations. Specific antibacterial activities of all currently known apidaecin-type peptides were also determined in liquid culture against seven selected bacterial strains (table 4). *E. coli* (wild type and resistant strains) and *S. typhimurium* were chosen as common laboratory strains, *Morganella* and *Yersinia* strains for their contrasting apidaecin-analog-antibiograms, and the *Acinetobacter* strain as a representative of moderately sensitive non-enterobacteriaceae. MIC's were first tested using bacterial cultures with inoculi of about 10^7 colony forming units (CFU) per ml; no significant killing activities (all MIC's > 40 µg/ml) were observed against the *Morganella* and *Yersinia* strains, and only peptides Cd2+, Cd3+ and Ho+ had measurable activities against *E. coli* 11775apid^R. For those bacterial strains, tests were repeated using an inoculum of 1-5x10^3 CFU/ml. This time, MIC's of several peptides were in the low- to sub-microgram/ml range. Thus, an inoculum effect (for definition, see Ref. 45) exists for apidaecin-type antibacterial peptides against certain strains, but not against all, as for example peptides Cd3+ and Ho+ inhibit viability of *E. coli* ATCC11775 equally well with inoculum sizes differing by as much as four orders of magnitude (table 4). No inoculum effect was observed for cecropin P1 with any of the bacterial strains used in this study.

MIC's of cecropin P1 against *E.coli* and *Morganella* strains were 0.2-1 µg/ml; this is in contrast to the absence of any measurable growth inhibition properties against the same strains on agar plates (table 4). Less dramatic discrepancies between low inhibitory activities on plates and higher ones in liquid culture were also observed for a few apidaecin-type peptides against some strains. While the reason for these discrepancies may be unclear at present, each observation of a strong inhibition zone has invariably been confirmed by low MIC

values in culture.

From the numbers presented in table 4, it follows that apidaecin-type peptides have antibacterial activities in the nanomolar to low micromolar range ($5x10^{-9}$ to $2.5x10^{-6}$M) against many *Enterobacteriaceae*; it has been shown previously that specific activities against plant-associated microbes are even better (10^{-8}M) (1,24). However, as was already clear from the plate tests, different peptides seem to be selectively better at growth inhibition, or killing, of different bacteria (e.g. Hb Ib against *E. coli* ATCC11775, Ho+ against the *Acinetobacter* strain and Cd2+ against *E. coli* 11775apid^R mutant strain).

Rescue Polypeptides

One polypeptide isolated from hornets (PAB-FT) (Table 5) has antibacterial activity despite a Proline to Threonine substitution in a highly-conserved region. Experiments were designed to determine whether the Proline-rich N-terminal region of PAB-FT is responsible for "rescuing" activity. Various artificial constructs were made (Table 5). Synthetic polypeptides having the Pro to Thr substitution but lacking the N-terminal proline-rich region (Ho-GT and Ho-FT) lack detectable antibacterial activity, but polypeptides with the N-terminal proline-rich region have antibacterial activity, as determined by their minimal inhibition zone. It was further found that antibacterial activity is rescued by a partial PAB-FT N-terminal region (Table 6).

Table V/Apidaecin related "rescue" peptides:

Peptide (alternative name)	Sequence	[M+H+]
Ho+ (=HoGP)	G K P R P Q Q V P P R P P H P R L	1958.33
PAB-GP	S R P S P Q V P I R P S Q P R P Q P G K P R P Q Q V P P R P P H P R L	3964.65
HoFP (=HoG1F)	F K P R P Q Q V P P R P P H P R L	2048.45
PAB-FP	S R P S P Q V P I R P S Q P R P Q P F K P R P Q Q V P P R P P H P R L	4054.77
HoGT (=HoP12T)	G K P R P Q Q V P P R T P H P R L	1962.32
PAB-GT	S R P S P Q V P I R P S Q P R P Q P G K P R P Q Q V P P R T P H P R L	3968.63
HoFT (=HoG1F/P12T)	F K P R P Q Q V P P R T P H P R L	2052.45
PAB-FT1-35	S R P S P Q V P I R P S Q P R P Q P F K P R P Q Q V P P R T P H P R L	4058.76
PAB-FT6-35	Q V P I R P S Q P R P Q P F K P R P Q Q V P P R T P H P R L	3534.18
PAB-FT12-35	S Q P R P Q P F K P R P Q Q V P P R T P H P R L	2843.33

(SEQ ID NOS.:32-41)

Of these peptides, only Ho+ and PAB-FT are naturally occurring in hornets; all others are artificial constructs.

Residues that differ from the Ho+ sequences are underlined (except the N-terminal extensions).

Masses are listed as average molecular mass [M] plus [+] 1 proton [H+]

Table VI: Antibacterial activities of apidaecin-type "rescue" peptides

BACTERIAL STRAINS	PEPTIDES Ho+	PAB-GP	HoFP	PAB-FP	HoGT	PAB-GT	HoFT	PAB-FT 1-35	PAB-FT 6-35	PAB-FT 12-35
E. coli ATCC11775	15	10	13	10	–	8	–	10	13	10
E. coli D22	14	14	16	14	–	13	–	14	14	10
Salmonella typhimurium	14	6	12	7	–	6	–	7	8	–
Salmonella typhi	20	13	13	13	–	12	–	14	14	5
Agrobacterium tumefaciens	12	8	11	7	–	5	–	5	ND	ND
Erwinia amylovora	12	5	11	5	–	6	–	5	ND	ND
Pseudomonas syringae	13	6	12	+	ND	5	–	–	ND	ND
Enterobacter cloacae	15	13	14	11	ND	9	ND	11	12	11
Klebsiella pneumoniae	15	9	14	8	–	8	–	9	11	6
Shigella dysenteriae	18	11	18	12	–	10	–	12	12	11
Acinetobacter calcoaceticus	5	9	–	8	–	8	–	7	10	–

Inhibition zones: in mm diameter
Peptides: 50 nanomoles per well (3mm diameter)
ND: Not determined

DISCUSSION

Apidaecin is a small peptide antibiotic (18 L-amino acids, unmodified), isolated from honeybees. It is lethal for many gram negative bacteria in nanomolar doses (1,24) and has a unique 'non-lytic' mechanism that involves stereo-selectivity (Casteels and Tempst, submitted); gram positives are nearly unaffected. Assessment of therapeutic prospects mandates the elucidation of mode-of-action and understanding of the functional role played by each component amino acid. It was decided to look at nature (i.e. evolution) to understand structure / function of this bioactive peptide. Here isolation and structural characterization of 13 novel, naturally occuring apidaecin-type peptides and functional analysis (antibacterial spectra) of 17 members of this class of antibiotics are reported.

The search for novel apidaecin-type peptides was greatly facilitated by the use of immuno-detection (using anti-apidaecin polyclonal antiserum) and mass spectrometric analysis (scanning for molecules in the expected molecular weight range) of liquid chromatographic fractions of induced insect lymph. This not only eliminated the need for tedious antibacterial assays but, most importantly, required consumption of only low nanogram (ELISA) to femtogram (MALDI-TOF MS) quantities of peptide. Typically, the amounts needed for antibacterial assays are on the average one to two orders of magnitude more (1,24). As micro-isolation (29,46), chemical sequencing (33,34) and combined chemical/MS sequencing (31) of small peptides (<25-30 amino acids) are now possible at low picomole levels, less than 50 nanogram of apidaecin-type peptides were required for elucidation of their structures. Thus, studies as described in this report can be undertaken with only a few and/or very small insects as source material. Since

the present effort was the first of its kind, antibacterial assays were carried out as a back-up to make sure 'apidaecin-like' peptides that didn't cross-react with the antiserum and, additionally, were unusually big or small would not be overlooked. While, in this way, several novel "other" peptides were discovered, no additional "apidaecin-type" ones were found. Thus, the approach taken here may be more widely applicable.

Sequence alignment of all currently known apidaecin-type peptides allowed delineation of strictly conserved 'core' sequences (figure 1). Because the strong reliance of insects on these peptides for survival, and considering the time-scale of divergence within the *Hymenoptera* order (27), evolutionary pressure on the conserved sequences must have been extraordinary. Hence, it is speculated that these 'core' sequences are essential for general antibacterial capacity. Consistent with this view was the observation that the only apidaecin-type peptide with a substitution in the 'core' sequence (Pro(9) to Ser in Hb III; figure 1) is a very poor antibiotic (little to none activity and very narrow spectrum; see tables 2 and 4). This peptide had been chemically synthesized, based on an isoform sequence observed in the open reading frames of some apidaecin cDNA clones. As speculated before (35), this peptide may actually not exist in nature as it is linked in the precursor to an unlikely processing sequence and because it has never been detected in insect lymph (24).

Given that, hitherto, apidaecins (from honeybees) lacked appreciable sequence similarities with other known polypeptides (in NBRF, PIR and SwissProt databases) and because of the presently established high degree of structural conservancy, it is clear that apidaecin-type peptides remain a very distinct group (17 members) of

antibacterial molecules. However, when introducing a few gaps for optimal alignment (in figure 2), a partial but unmistakable sequence match could be delineated between hornet apidaecin (Ho+) and drosocin from *Drosophila* (2). The *Drosophila* peptide however, is glycosilated whereas hornet apidaecin is not. Other Arg-Pro-rich antibacterial peptides have been described, namely insect abaecin (42) and mammalian Bac 5 and 7 (47), and PR-39 (44). None of those can be aligned too easily with apidaecins and they are distinctively lacking in histidine, a conserved residue among apidaecin-type peptides and also present in drosocin (figures 1, 2).

Analysis of biological activities indicated striking differences in antibacterial spectra between several apidaecin-type peptides (tables 2, 3). Structural elements underlying these differences must be located within small stretches of variable amino acid sequence as all apidaecin-type peptides show a high degree of conservation. Thus, functional variability among apidaecin-type peptides could be mapped to just a few amino acids. While this has also been observed for cecropin and magainin antibacterial activities, albeit investigated by comparing artificially designed synthetic analogs (48-50), in those instances, differences were exclusively of a quantitative nature (i.e. higher/lower MIC's against the same strains and as a result, wider/narrower spectra). In contrast, subtle amino acid substitutions in apidaecin-type peptides shift the antibacterial spectrum to a sometimes mutually exclusive pattern. For instance, replacing both Arg and Gln (at positions 4 and 10 in the sequence) with lysines (K/K at 4/10) results in a dramatic decrease of apidaecin activity against *Salmonella* and completely abolishes activity against *Yersinia* under the test conditions used; yet, these very changes create a peptide that is highly lethal for *Campylobacter jejuni* and *Legionella*

pneumophila, whereas the R/Q-variant was totally ineffective (table 3A). More examples of specificity-determining motifs can be found in table 3. As these motifs are fully contained within the spacer-sequences that separate conserved domains, it seems justified to advance the hypothesis that apidaecin-type peptides consist of 'constant' regions, conferring general antibacterial capacity (i.e. any modifications to these would abolish all function), and 'variable' regions, determining specificity (i.e. antibacterial spectra). Functional variability of these peptides is further highlighted by the remarkable finding that apidaecin-analog-antibiograms of certain bacteria (see table 2) are nearly 'mirror-image' of one another (e.g. *Yersinia* and *Campylobacter*), a phenomenon unique among all peptide antibiotics described so far.

The explicit finding that apidaecins antibacterial spectra can be manipulated has inherent practical consequences, i.e. creating peptides with a wider or a targeted narrower spectra. Wider antibacterial spectra can be most easily obtained by administration of several peptides (e.g. HbIb plus Ho+ plus Cd3+) simultaneously. As an alternative, multipotent single molecules may conceivably be constructed by combinatorial shuffling and/or point-substitutions of 'variable' regions, with the restriction however, that some specific killing activities are mutually exclusive. Similarly, designer narrow-spectrum peptides could be derived in this way. Thus, the 'constant/variable region' model will guide the future apidaecin-analog synthesis and testing program. While the significance of broad-spectrum antibacterial chemotherapeutics is generally appreciated, developing a narrow-spectrum drug is less conventional. However, from the data presented here, the synthesis in the not too distant future of an antibacterial drug specific for *Campylobacter jejuni* is envisioned. This microorganism

is a food-borne (raw milk) enteric pathogen, causing an estimated 2 million infections in the U.S. each year, and speculated to be a more frequent source of diarrhea in humans than *Salmonella* and *Shigella* (51). A specific antibiotic might be useful to treat such infections without affecting the patient's enteric flora (mostly *Enterobacterioceae*). Along the same lines, an apidaecin-type peptide that would be rationally modified to target plant pathogens exclusively (a goal that doesn't seem overly complicated to attain) could be engineered into transgenic crops, for antimicrobial protection, without affecting man or animal that eat them.

Drug resistance is a major problem in antibacterial chemotherapy (20). Apidaecin could therefore only be considered for clinical applications after suitable 'back-up' peptides have been identified or developed. By virtue of complementarity, four different types of inducible peptides confer broad-spectrum antibacterial defense to honeybees (1). Among those, apidaecins provide protection against the majority of gram negative infections. Evolutionary, potential problems with apidaecin-resistance may have been countered by the synthesis of a second Arg-Pro-rich peptide, abaecin, that is otherwise different in sequence and substantially less active than apidaecin. It has previously been shown that the inhibitory effects of abaecin on an apidaecin-resistant *E. coli* strain (mutant 11775apid^R) are unattenuated as compared to the wild-type strain (42). Effective as abaecin may be for coping with sporadic resistance in honeybees, it is probably too low in specific activity to substitute for the highly potent apidaecin in cases like nosocomial infections.

Results of the current study prove that small sequence changes to apidaecin itself also allow to overcome emerging antibacterial resistance (see tables 2 and 4).

Furthermore, a Gly-Lys-Pro sequence at the amino-terminus critically determines activity of peptide Ho+ against apidaecin-resistant strain 11775apid^R, as removal of this triplet abolishes lethal capacity against the mutant but not against the parental strain. These observations, together with the fact that mutant and parent strain are equally susceptible (resistant) to 'lytic' peptides (e.g. cecropin P1, magainin B) (25), refute the notion that the emerging apidaecin-resistance was the result of a simple barrier mutation (i.e. physically excluding passage of antibiotic through the cell enveloppe). Barrier-mutations, affecting outer membrane structure and/or protein content, have typically been implicated in bacterial resistance to lytic peptides. For example, resistance of *Salmonella* to polymixin B action has been correlated with an altered *pmrA* gene (52).

The present findings are therefore in keeping with earlier observations that apidaecin-type peptides exert antibacterial activities through a non-lytic mechanism, involving stereoselective interactions (25). The total picture emerging is consistent with the view that specific molecular recognition, between peptide and bacterial "receptor/docking" molecule(s), underlies lethal function of apidaecin-type peptides. Strain-specific variation (sequence differences) and novel alterations (mutations) of these chiral 'target-molecules' might constitute the molecular basis for, respectively, drug-specificity and -resistance. This speculation is founded in the known *E.coli* quinolone-resistance mechanisms (53), whereby various selected alterations in DNA gyrase result in differential 'resistance' phenotypes (e.g. the same point mutation causing sensitivity to nalidixic acid to go down but that to the structurally related norfloxacin to go up). Conceivably then, combinations of several apidaecin-type peptides, with diverse 'variable' regions, may prevent

resistance from easily emerging.

Finally, while 'target-molecules' could reside on the bacterial surface, the notion that, after apidaecin binding, a lethal 'signal' would be transmitted to upset vital structures or functions inside the cell, is hard to comprehend. In addition, with a molecular weigth of over 2,000 dalton, apidaecin is probably too big to utilize the outer membrane porins for passive influx. Then how would the peptide get into the cells? Colicins (antibacterial proteins of bacterial origin) are known to do so by utilizing host membrane receptors (e.g. for vitamin B12 or nucleotides) for inward translocation (54); once inside, they interact with a second target molecule, causing cessation of vital cell function or death. It is a provocative thought that apidaecins might do the same.

Second Series of Experiments
Use of Apidaecin-Like Antimicrobials

Large scale extraction of apidaecin from immune insects appears unrealistic. Instead, the peptides can be manufactured by bulk chemical synthesis. Alternatively, they could be produced in yeast (which is not among the apidaecin targets) by constructing multicopy plasmids, containing a fusion gene of apidaecin and the yeast α mating factor (αMF) promotor and secretion signals (Reichhart et al, 1992; Martin-Eauclaire et al, 1994). Apidaecin precursors contain multiple (up to 12) peptides which, interestingly, are assembled and thought to be processed in exactly the same way as yeast αMF (Casteels-Josson, 1993). Efficient biological production in this system appears therefore very likely. Ample possibilities exist for useful, economically justified applications in several areas of antimicrobial chemotherapy: i) use against multiple-antibiotic-resistant strains, ii) antimicrobial combination chemotherapy, and iii) against target organisms of high pathogenic potential.

Multiple-antibiotic-resistance. Since the early penicillin days, it has been realized that certain bacteria were not killed by antibiotics. Resistance to specific (or groups of) antibiotics can be intrinsic or, more often, is acquired; both types of resistance complicate treatment of infection. Spread of multiple-antibiotic-resistance (MAR) among bacteria is commonly associated with the exchange of plasmids (Foster, 1983; Davis, 1994). In addition, chromosomal MAR can be derived from exposure of susceptible cells to low concentrations of antimicrobial drugs (Hachler et al, 1991). Either way, the emergence of multi-drug resistance is a major concern for physicians and

pharmaceutical industry, and the problem is worsening rapidly (Science 264:359-393). Antimicrobials that are structurally unrelated to any of the other recognized antibiotic groups, and act via novel mechanisms, may be effective therapeutics when everything else fails. For instance, resistance to mupirocin, a competitive inhibitor of the isoleucine transfer-RNA synthase (and therefore of protein synthesis) is extremely uncommon (Hughes and Mellow, 1978; Russell and Chopra, 1990); Neu, 1991). Thus, when such a novel antibiotic is not extensively prescribed, it would be available as "drug of the last resort".

Antimicrobial combination chemotherapy. Antibiotic combinations are frequently used to i) achieve broad-spectrum empiric coverage in critically ill patients with undefined bacterial infections, ii) treat mixed bacterial (polymicrobial) infections, with strains that may not have a common antibiotic susceptibility, iii) prevent emergence of resistance against a single antibiotic, and because iv) the identified pathogen has been typed as resistant to inhibition/killing by a conventional dose of any single antibiotic, but a combination may achieve a synergistic effect (Rahal, 1978; Eliopoulos and Moellering, 1991). The latter scenario may result in a decrease of dose-related toxicity and is therefore, as well as the other instances of combination treatments, of great clinical relevance.

As regards possible synergisms of apidaecin and apidaecin-like polypeptides with "classical" drugs, the following three uses are provided: i) sequential inhibition of a common biochemical pathway (as for instance the case of trimethoprim+sulfomethoxazole effects on the bacterial tetrahydrofolate synthesis pathway (Poe, 1976)), ii) sequential inhibition of cell wall synthesis (in combination with, for example, beta-

lactam antibiotics, or with vancomycin), and iii) the use of beta-lactam or other agents that act on the cell wall to facilitate apidaecin entry. Many published accounts are available on synergistic combinations 'in-vitro' (Farber et al, 1986; Hackbarth et al, 1986; Bustamante et al, 1987; Whiting et al, 1987), and were prognostic for 'in-vivo' outcome in some cases (Chandrasekar et al, 1987). However, possible antagonistic effects may occur, as for instance when i) combining bacteriostatic agents with beta-lactam antibiotics (Brown and Alford, 1984), or aminoglycosides, or quinolones (Zeiler, 1985), and ii) combining protein synthesis inhibitors that act on the 50S ribosome (Eliopoulos and Moellering, 1991).

Target organisms of higly pathogenic potential. The majority of *Enterobacteriaceae* and *Haemophilus influenzae* are among the prefered targets of apidaecin (Casteels et al, 1993, 1994; see also preliminary results section). Specific strains from several species belonging to this family and of *H. influenzae* have caused hospital bacteremias (Balows et al, 1991), recurring resistance problems (among numerous cases: *Salmonella* (Holmberg et al, 1984), *Shigella* (Ling et al, 1988), *Citrobacter* (Gootz et al, 1984 and *Haemophilus* (Campos et al, 1989)), and pose serious risk factors for infection of the urban community (such as *E.coli* 0157:H17 (Bryant et al, 1989)).

One of the most sensitive bacterial species to lethal apidaecin activity is *Campylobacter jejuni* (Casteels et al, 1994; Fig. 2). The susceptibility of the strain that was tested rivals that of plant-associated bacteria, about one to two orders of magnitude more sensitive than most *Enterobacteriaceae*. Interestingly, *Campylobacter* sensitivity was peptide dependent, as only the Cd-apidaecin isotypes showed this remarkable activity (all others were totally inactive). It seems that the precise structural requirements of apidaecin for specific

activity against this species have been unlocked. A very closely related species (at one point even refered to as "*Campylobacter*") is *Helicobacter pylori*, the major etiological agent of chronic gastritis and peptic ulcer disease (Marshall, 1990, 1993; Blaser, 1990a, 1990b, 1992). Standard therapy that heals duodenal ulcers, colliodal bismuth subcitrate (CBS), also inhibits growth of *H. pylori* in vitro but does not eradicate the organism (Marshall, 1993). It is now common to add other, selected antibiotics (that show inhibition of *H. pylori* in their own right) to the bismuth regimens. Examples of such agents are metronidazole (DeCross et al, 1993) and clarithromycin (Peterson et al, 1993), but developing antibiotic resistance has been observed (Marshall, 1993). Others include amoxicillin, tetracyclin, furazolidone and nitrofurantoin, and many combination have been tried therpeutically, all or not with bismuth (Marshall, 1993)

RESULTS

Apidaecin antibacterial activity in serum:

Stability and protein-binding in serum. Apidaecin, when used therapeutically, would come in conact with serum components, and bound peptide would not be available for antimicrobial action. The protein-binding and possible degradation of apidaecin in fetal bovine serum were investigated. To this end, peptide was incubated in serum (or water for the controls) for various time periods. Levels of free, undegraded peptide were then measured by RP-HPLC, after prior removal of proteins by ultrafiltration (Centriprep system). This technique allows to remove protein-bound peptide (Gootz et al, 1988) and, in addition, to monitor partial degradation of free peptide simultaneously; large fragments can be detected by HPLC. The results in table 7 show that at time zero, only 40% of the peptide (added in a 25 μg/ml

concentratrion) is recovered from the serum, indicating that about 60% must be protein-bound as the elapsed time was too short for measurable degradation. During the next three hours, approximately the same percentage of unbound peptide was recovered, further indicating absence of

Table 7. Protein-binding and degradation of apidaecin (isotype Ib) in serum:

Apidaecin was incubated in fetal bovine serum at 37°C, after which the mixture was deproteinated by ultrafiltration and the peptide levels in the filtrate determined by RP-HPLC analysis.

A: apidaecin Ib (25 µg/ml serum) was incubated for various periods of time and the levels of free (undegraded) peptide measured (expressed as % of apidaecin-recoveries from incubation in water for similar time periods). Relative recoveries (%) are as compared to the time zero value.

B: various concentrations of apidaecin Ib were added to serum and immediately (T=0) subjected to ultrafiltration and RP-HPLC analysis. Levels of free apidaecin are expressed as % of recoveries from solutions in water at the same concentrations. Relative recoveries (%) are as compared to the 100 µg/ml.

A	Time apidaecin relative to T=0		B	Concentration apidaecin relative to 100 µg	
	recovery			recovery	
0 '	39%	100%	100 µg/ml	47%	100%
30 '	38%	97%	25 µg/ml	42%	89%
3 h	39%	100%	5 µg/ml	56%	119%
24 h	21%	54%			

early-immediate proteolysis. After 24 h, levels had been further reduced by 50% (20% of peptide originally added to serum). Thus, the half-life of apidaecin in serum is about 24 h (corrected for binding). Incubation of various concentrations (5-100 µg/ml) apidaecin in serum for less than 1 min, indicated that peptide binding is strictly proportional to the amount added (about 40-50% in all cases) and that low concentrations are not more esily "sopped up". Taken together, it does not appear that serum levels of apidaecin will drop excedingly fast below effective concentrations.

Effect of serum on apidaecin in-vitro MICs. MICs were determined for apidaecin (isotype Ib) against five clinical isolates (*E.coli* and *Enterobacter*), both in 100% cation adjusted Mueller-Hinton (CAMH) broth and in 50% fetal bovine serum (+50% CAMH broth). All five strains grew well in 100% serum in the absence of antibiotics, indicating their resistance to serum bactericidal components (data not shown). Two of the isolates (Enterobacters) were selected because of their moderate-to-high resistance to many penicillins and cephaloporins (listed in table 8). The results indicate that by addition of serum, apidaecin MIC either stays the same (H16 strain) or increases by a factor of 2-4 (table 8). Serum protein binding (see above) may account for all of this apparent decrease in activity. For comparison, MICs of magainin B and cecropin P1 to inhibit strain H16 in serum were, respectively, 200 and 25 µg/ml (same as apid). Limited experiments with rabbit and human serum gave essentially the same results. Thus, apidaecin-type peptides are fully active in serum.

Table 8. Apidaecin (Ib) MIC's in broth and serum:

All bacterial strains are clinical isolates and grow very well in 100% serum (data not shown). MIC values (in µg/ml; from triplicate tests) of apidaecin for each

strain were determined in 96-well microtiter plates, with peptide concentrations ranging from 200 to 0.1 µg/ml. Cells (1-5 x 105 cfu / ml) were inoculated in broth (Cation Adjusted Mueller-Hinton) or in 50% fetal bovine serum (in broth), incubated at 37°C, and wells scored for growth after 24 h. Sloan-Kettering (MSK) isolates were tested for sensitivity to penicillins and cephalosporins, using Microscan PMP Neg MIC panels: strain N548 is non-resistant to all of the antibiotics tested; N541 and N661 are moderately to strongly resistant to piperacillin (+ tazobactam), ceftriaxone, ceftazidime, cefotetan, cefuroxime, and N661 additionally to ticarcillin (+ clavulonic acid), ampicillin (+ sulbactam), cefotaxime and cefoxitin.

Strain	Strain I.D. (source)	100% CAMH	50% serum / 50%CAMH
		µg/ml	µg/ml
E. coli	25922 (ATCC)	6.25	25
E. coli	H16 (Darveau)	25	25
E. coli	N548 (MSK)	6.25	12.5
Enterobacter sp.	N541 (MSK)	25	100
Enterob. aerogenes	N661 (MSK)	50	100

Absence of apidaecin toxicity and antigenicity to animals (and cells):

Investigations were carried out on possible apidaecin toxicity and antigenicity in higher animals, traits that are highly undesirable for an antibiotic to be considered of practical use. No visible detrimental effects occured when mouse spleen cells were cultured (RPMI medium with 5% fetal calf serum), for four days, in the presence of 5 microgram/ml of the peptide (unpublished data). Since apidaecin is rather small, a very weak immune response could be elicited in mice only when the peptide was injected in the presence of Freund's adjuvant, not by itself. Moreover, for adequate immunization of rabbits

with apidaecin, the peptide had to be coupled to tuberculin (from *Mycobacterium tuberculosis*; heat-inactivated protein obtained from Cambridge Research Pharmaceuticals) and the rabbits prevaccinated (with live *Bacillus Calmette-Guerin*) three weeks before injection (Casteels et al, 1994). In this way, and in this way only, was anti-apidaecin antiserum obtained; again, by itself, apidaecin was a very poor antigen.

Growth inhibition of clinical isolates by apidaecin:

In an earlier screen, only one strain per bacterial species, for the most part not clinical isolates, had been tested. Those efforts have been extended to a considerable larger number of clinical isolates. All isolates had been tested for sensitivity to classical antibiotics. Apidaecins Ib, Ho+ and Cd3+ were selected as representatives of the three groups of isotypes, and used in agar zone inhibition tests. Results are shown in table 9. A very large member of enteric bacteria were sentive to the growth inhibiting properties of the apidaecins. Not unexpectedly, the effects on *Pseudomonas aeruginosa* were minimal (only 4 out of 29 isolates were sensitive); 20 gram-positive isolates were also refractory to apidaecin activitiues. On the other hand, about half of the *Proteus mirabilis* isolates were growth-inhibited, in contrast to the earlier observation that a single test strain (ATCC25933) was resistant to all apidaecins (Casteels et al, 1994). In conclusion, if not counting *Pseudomonas*, about 85% of all clinical isolates were sensitive targts for antibacteial activities.

Table 9. Growth inhibition of clinical isolates by apidaecins (in disk diffusion tests):

Bacterial strains were isolated from patients at Memorial Hospital (MSK) and made available by the Infectious Disease Service. They were tested for sensitivity to

classical antibiotics (Microscan PMP Neg MIC panels) and to apidaecin, isotypes Ib, Ho+ and Cd3+ (using the agar inhibition zone test with 100 µg peptide per 6.35-mm disk). No tests were carried out on gram-positives.

Bacterial strains	Total number tested	Sensitive to apidaecin (isotype):		
		Ib	Ho+	Cd3+
	n	*n*	*n*	*n*
E. coli	40	40	40	40
Klebsiella, all species	23	23	23	22
K. pneumoniae	20	20	20	19
K. oxytoca	3	3	3	3
Proteus mirabilis	11	6	5	6
Pseudomonas aeruginosa	29	4	4	4
Haemophilus influenzae	4	4	4	4
Enterobacter, all species	19	19	19	19
E. cloaca	12	12	12	12
E. aerogenes	7	7	7	7
Morganella morganii	5	0	0	0
Citrobacter freundii	13	13	13	12
Serratia marcescens	6	0	0	0
Total	150	109	108	107
Without *Pseudomonas*	121	105 (87%)	104 (86%)	103 (85%)

Preventing and combating acquired resistance:

To guarantee long-term usage of a new antimicrobial drug, steps must be taken in anticipation of the inevitable emergence of resistance, i.e. by development of second generation analogs. Spontaneous resistant mutants against apidaecin Ib, derived from *E. coli* strain ATCC11775 (one mutant) and ATCC25922 (two mutants) have been observed. Subtle well-positioned changes in the apidaecin amino acid sequence allow negation of this

acquired resistance. Indeed, all three mutants are still quite sensitive to isotype Cd3+, and two are sensitive to isotype Ho+. Evidence exists that the mutant phenotypes are not of the "barrier" type (Casteels and Tempst, 1994; Casteels et al, 1994). It is highly conceivable that spontaneous resistance would emerge far less frequently on a mixed background of active peptides (with different sequences), as this would require multiple, specific mutations within the span of one to a few generations.

Evaluation of apidaecin antimicrobial combinations:

The merits and pitfalls of antimicrobial combination chemotherapy have been discussed above. Possible apidaecin potentiating effects on classical antibiotics (or vice versa) have been explored. Beta-lactam compounds were slected for initial trials, as it was speculated that they might alter the bacterial cell enveloppe ("antibiotic-injury") in such a way that increased entry of apidaecins would be facilitated. BAXTER MICROSCAN PMP Neg Combo panels were used, to which various concentrations of apidaecin Ib (1/2-1/16MIC) were added. Two clinical isolates were tested, one relative sensitive and the other moderate-to-high resistant to the drugs in the panel. Table 10 contains a partial listing of the results, indicating that the MICs for some cephalosporins / penicillins in broth and serum were reproducibly reduced by a factor of two by addition of apidaecin (at concnetrations of 1/2-1/4MIC). This constitutes a substantial reduction in drug concentration in some cases (e.g. from 64 to 32 μg/ml for the MIC of cefotetan to inhibit growth of strain N661). Judging from the results, there is certainly no indication of major antagonisms, which would allow combination therapy to prevent emergence of drug-resistant sub-populations of a pathogenic organism.

Table 10. Growth inhibition of clinical isolates by combinations of apidaecin (Ib) and penicillins / cephalosporins in broth and serum:

A: MIC values (µg/ml) for apidaecin, MagB (magainin antibacterial peptide from frogs) and CecP1 (cecropin antibacterial peptide from pig intestine); CAMH is cation adjusted Mueller-Hinton broth.

B: Effect of apidaecin (at 1/2 and 1/4 of its MIC value) on the MIC's of penicillins and cephalosporins;

"x : y" denotes MIC values (of classical antibiotics) without (x) or with (y) apidaecin.

All tests in triplicate.

Abbreviations: A/S, ampicillin (+sulbactam); TIM, ticarcillin; Pi, piperacillin; P/T, piperacillin (+tazobactam); CFX, cefoxitin; CRM, cefuroxime; CAX, ceftriaxone; CFT, cefotaxime; CTN, cefotetan.

Table 10 continued

	Growth medium	apidaecin	other	*E. coli* H16 (4x10³cfu/ml)	*Enterobacter* N661 (5x10⁵cfu/ml)
				MIC (µg/ml)	*MIC (µg/ml)*
A	100% CAMH	+	-	20	40
	50% serum/broth	+	-	20	100
	"	"	MagB	400	n.d.
	"	"	CecP1	100	n.d.
				w/o : with apid	w/o : with apid
B		*µg/ml*		*MIC (µg/ml)*	*MIC (µg/ml)*
	E. coli H16				
	100% CAMH	10 (1/2MIC)	A/S	2 : <1	
	"	"	CFX	2 : <1	
	50% serum/broth	10 (1/2MIC)	CFX	4 : <1	
	"	"	CRM	4 : 2	
	"	"	TIM	2 : <1	
	Enterobacter N661				
	100% CAMH	10 (1/4MIC)	CAX		8 : 4
	"	20 (1/2MIC)	"		8 : 4
	"	10 (1/4MIC)	Pi		32 : 16
	"	20 (1/2MIC)	"		32 : 16
	"	"	CFT		8 : 4
	"	"	CTN		64 : 32
	"	"	P/T		8 : 4
	50% serum/broth	50 (1/2MIC)	CAX		8 : 4

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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TEMPST, PAUL
CASTEELS, PETER

(ii) TITLE OF INVENTION: APIDAECIN-TYPE PEPTIDE ANTIBIOTICS WITH IMPROVED ACTIVITIES AND/OR DIFFERENT ANTIBACTERIAL SPECTURM

(iii) NUMBER OF SEQUENCES: 43

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.24

(vii) PREVIOUS APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/205,938
(B) FILING DATE: 02-MAR-1994
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 8
(D) OTHER INFORMATION: /note= "Residue 8 is Ile or Leu."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Pro Arg Pro Pro His Pro Arg Xaa
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..5
(D) OTHER INFORMATION: /note= "Residue 1 is Arg or Lys.
Residue 3 is Thr, Gln or Arg.
Residue 4 is Tyr, Gln or Pro.
Residue 5 is Val or Ala."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Xaa Pro Xaa Xaa Xaa Pro
1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Arg Pro Thr Tyr Val Pro
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Arg Pro Gln Gln Val Pro
1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Arg Pro Arg Pro Ala Pro
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Lys Pro Arg Pro Ala Pro
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Asn Arg Pro Thr Tyr Val Pro Pro Pro Arg Pro Pro His Pro Arg Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Asn Arg Pro Thr Tyr Val Pro Ala Pro Arg Pro Pro His Pro Arg Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gly Lys Pro Arg Pro Gln Gln Val Pro Pro Arg Pro Pro His Pro Arg Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Arg Pro Gln Gln Val Pro Pro Arg Pro Pro His Pro Arg Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ser Asn Lys Pro Arg Pro Gln Gln Val Pro Pro Arg Pro Pro His Pro Arg Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Asn Lys Pro Arg Pro Gln Gln Val Pro Pro Arg Pro Pro His Pro Arg Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Gly Lys Pro Asn Arg Pro Arg Pro Ala Pro Ile Gln Pro Arg Pro Pro
1 5 10 15

His Pro Arg Leu
20

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Asn Arg Pro Arg Pro Ala Pro Ile Gln Pro Arg Pro Pro His Pro Arg Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Gly Lys Pro Asn Lys Pro Arg Pro Ala Pro Ile Lys Pro Arg Pro Pro
1 5 10 15

His Pro Arg Leu
20

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Asn Lys Pro Arg Pro Ala Pro Ile Lys Pro Arg Pro Pro His Pro Arg Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Gly Lys Pro Ser Lys Pro Arg Pro Ala Pro Ile Lys Pro Arg Pro Pro
1 5 10 15

His Pro Arg Leu
20

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Ser Lys Pro Arg Pro Ala Pro Ile Lys Pro Arg Pro Pro His Pro Arg Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /note= "Residue 8 is Ile or Leu."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Pro Arg Xaa Pro His Pro Arg Xaa
1 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Ser Gln Pro Arg Pro Gln Pro
1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Gln Val Pro Ile Arg Pro Ser Gln Pro Arg Pro Gln Pro
1 5 10

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Ser Arg Pro Ser Pro Gln Val Pro Ile Arg Pro Ser Gln Pro Arg Pro Gln Pro
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Gly Asn Asn Arg Pro Val Tyr Ile Pro Gln Pro Arg Pro Pro His Pro
1 5 10 15

Arg Ile

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Gly Asn Asn Arg Pro Val Tyr Ile Pro Gln Pro Arg Pro Pro His Pro
1 5 10 15

Arg Leu

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Gly Asn Asn Arg Pro Ile Tyr Ile Pro Gln Pro Arg Pro Pro His Pro
1 5 10 15

Arg Ile

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Gly Asn Asn Arg Pro Val Tyr Ile Ser Gln Pro Arg Pro Pro His Pro
1 5 10 15

Arg Ile

(2) INFORMATION FOR SEQ ID NO:27:

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Ala Asn Arg Pro Val Tyr Ile Pro Pro Pro Arg Pro Pro His Pro Arg Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Asn Arg Pro Val Tyr Ile Pro Pro Pro Arg Pro Pro His Pro Arg Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Gly Lys Pro Arg Pro Gln Gln Val Pro Pro Arg Pro Pro His Pro Arg Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Gly Lys Pro Arg Pro Tyr Ser Pro Arg Pro Thr Ser His Pro Arg Pro
1 5 10 15

Ile Arg Val

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Pro Arg Pro Pro His Pro Arg Leu
1 5

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Gly Lys Pro Arg Pro Gln Gln Val Pro Pro Arg Pro Pro His Pro Arg Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Ser Arg Pro Ser Pro Gln Val Pro Ile Arg Pro Ser Gln Pro Arg Pro
1 5 10 15

Gln Pro Gly Lys Pro Arg Pro Gln Gln Val Pro Pro Arg Pro Pro His
20 25 30

Pro Arg Leu
35

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Phe Lys Pro Arg Pro Gln Gln Val Pro Pro Arg Pro Pro His Pro Arg
1 5 10 15

Leu

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Ser Arg Pro Ser Pro Gln Val Pro Ile Arg Pro Ser Gln Pro Arg Pro
1 5 10 15

Gln Pro Phe Lys Pro Arg Pro Gln Gln Val Pro Pro Arg Pro Pro His
20 25 30

Pro Arg Leu
35

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Gly Lys Pro Arg Pro Gln Gln Val Pro Pro Arg Thr Pro His Pro Arg Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Ser Arg Pro Ser Pro Gln Val Pro Ile Arg Pro Ser Gln Pro Arg Pro
1 5 10 15

Gln Pro Gly Lys Pro Arg Pro Gln Gln Val Pro Pro Arg Thr Pro His
20 25 30

Pro Arg Lys
35

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Phe Lys Pro Arg Pro Gln Gln Val Pro Pro Arg Thr Pro His Pro Arg Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Ser Arg Pro Ser Pro Gln Val Pro Ile Arg Pro Ser Gln Pro Arg Pro
1 5 10 15

Gln Pro Phe Lys Pro Arg Pro Gln Gln Val Pro Pro Arg Thr Pro His
20 25 30

Pro Arg Leu
35

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

Gln Val Pro Ile Arg Pro Ser Gln Pro Arg Pro Gln Pro Phe Lys Pro
1 5 10 15

Arg Pro Gln Gln Val Pro Pro Arg Thr Pro His Pro Arg Leu
20 25 30

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

Ser Gln Pro Arg Pro Gln Pro Phe Lys Pro Arg Pro Gln Gln Val Pro
1 5 10 15

Pro Arg Thr Pro His Pro Arg Leu
20

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

Ser Asn Lys Pro
1

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 4
(D) OTHER INFORMATION: /note= "Residue 4 is Asn or Ser."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

Gly Lys Pro Xaa
1

What is claimed is:

1. A purified polypeptide having antibacterial activity comprising

a first sequence Pro-Arg-Pro-Pro-His-Pro-Arg-X1 (SEQ ID NO: 1), wherein

X1 is Ile or Leu; and

a third sequence X2-Pro-X3-X4-X5-Pro (SEQ ID NO: 2), wherein

X2 is Arg or Lys,

X3 is Thr, Gln or Arg,

X4 is Tyr, Gln or Pro, and

X5 is Val or Ala,

the third sequence is N-terminal to the first sequence.

2. The polypeptide of claim 1 comprising up to about thirty-five amino acid residues.

3. The polypeptide of claim 2 comprising from about fourteen to about twenty-one amino acid residues.

4. The polypeptide of claim 1 wherein the third sequence is separated from the first sequence by up to two amino acid residues.

5. The polypeptide of claim 1, the third sequence is selected from the group consisting of

Arg-Pro-Thr-Tyr-Val-Pro (SEQ ID NO: 3),
Arg-Pro-Gln-Gln-Val-Pro (SEQ ID NO: 4),
Arg-Pro-Arg-Pro-Ala-Pro (SEQ ID NO: 5), and
Lys-Pro-Arg-Pro-Ala-Pro (SEQ ID NO: 6).

6. The polypeptide of claim 1, further comprising a fourth sequence selected from the group consisting of Gly-Lys-Pro and Asn-Lys-Pro, and Phe-Lys-Pro; wherein the fourth sequence is N-terminal to the second sequence.

7. The polypeptide of claim 6 wherein the fourth sequence is separated from the third sequence by up to two amino acid residues.

8. The polypeptide of claim 6 wherein the third sequence is selected from the group consisting of
Arg-Pro-Gln-Gln-Val-Pro (SEQ ID NO: 4),
Arg-Pro-Arg-Pro-Ala-Pro (SEQ ID NO: 5), and
Lys-Pro-Arg-Pro-Ala-Pro (SEQ ID NO: 6).

9. A purified polypeptide having antibacterial activity, comprising:
a first sequence Pro-Arg-Pro-Pro-His-Pro-Arg-(Ile/Leu) (SEQ ID NO.:1);
optionally, a second sequence immediately adjacent to the N-terminal amino acid residue of the first sequence, wherein the second sequence is selected from the group consisting of:
Pro;
Ala;
Ile-Gln; and
Ile-Lys;
a third sequence immediately adjacent to the second sequence, or immediately adjacent to the N-terminal amino acid residue of the first sequence when the polypeptide does not contain a second sequence, wherein the third sequence has the following formula:
X2-Pro-X3-X4-X5-Pro (SEQ ID NO: 2), wherein
X2 is Arg or Lys;
X3 is Thr, Gln or Arg;
X4 is Tyr, Gln or Pro; and
X5 is Val or Ala;
a fourth sequence immediately adjacent to the third sequence, wherein the fourth sequence is selected from the group consisting of:
Asn;

Gly-Lys-Pro;
Ser-Asn-Lys-Pro (SEQ ID NO.:42); and
Gly-Lys-Pro-(Asn/Ser) (SEQ ID NO:43);
wherein the fourth sequence is truncated by zero to four amino acid residues at its N-terminus;
and wherein the number of amino acid residues in the polypeptide is the sum of the number of second sequence residues, the number of fourth sequence residues, and fourteen.

10. The polypeptide of claim 1 comprising a sequence selected from the group consisting of:
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 7);
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Ala-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 8);
Gly-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 9);
Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 10);
Ser-Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 11);
Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 12);
Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13);
Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 14);
Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15);
Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 16);
Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17); and
Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 18).

11. The polypeptide of claim 1 selected from the group consisting of:
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 7);
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Ala-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 8);
Gly-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 9);
Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 10);
Ser-Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 11);
Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 12);
Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13);
Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 14);
Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15);
Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 16);
Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17); and
Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 18).

12. DNA encoding the polypeptide of claim 1.

13. A purified polypeptide having antibacterial activity isolatable from an insect;

the insect is selected from the group consisting of Sphecius speciosus, Vespula maculata, Vespula maculifrons, Paravespula germanica, and Coccygomimus disparis;

the polypeptide characterized by binding to an anti-apidaecin antibody.

14. A purified antibody capable of binding to the polypeptide of claim 1.

15. The antibody of claim 14 wherein the antibody is a polyclonal antibody.

16. A method for obtaining the purified antibody of claim 14 comprising:

coupling an antigen selected from the group consisting of honeybee apidaecin and the polypeptide of claim 1 to a carrier protein;

immunizing a mammal with the coupled antigen; and

isolating the antibody from the mammal, thereby obtaining the purified antibody.

17. The method of claim 16 wherein the carrier protein is tuberculin purified protein derivative.

18. The method of claim 16 wherein the immunizing is immunizing by injecting.

19. The method of claim 16 wherein the mammal is a rabbit.

20. A method for determining the presence of the polypeptide of claim 1 in a sample comprising

incubating the sample with the antibody of claim 14, and

detecting an antibody-antigen complex, thereby determining the presence of the polypeptide in the sample.

21. The method of claim 20 wherein the detecting comprises detecting by enzyme-linked immunoassay.

22. A method for inhibiting growth of a bacterium

comprising administering to the bacterium a growth inhibiting effective concentration of the polypeptide of claim 1.

23. The method of claim 22 for inhibiting growth of a bacterium selected from the group consisting of

Escherichia coli, Enterobacter cloacae, and Erwinia amylovora, Klebsiella pneumoniae, Salmonella typhimurium, Shigella dysenteriae, and Pseudomonas syringae

comprising administering to the bacterium a growth inhibiting effective concentration of a polypeptide of claim 11.

24. The method of claim 23 for inhibiting growth of an apidaecin resistant strain of Escherichia coli comprising administering a growth inhibiting effective concentration of a polypeptide selected from the group consisting of:

Gly-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 9);

Ser-Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 11);

Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 12);

Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13);

Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 14);

Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15);

Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 16);

Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17); and

Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 18).

25. The method of claim 22 for inhibiting growth of Morganella morganii comprising administering a growth inhibiting effective concentration of a polypeptide selected from the group consisting of:
Gly-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 9);
Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 10);
Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13);
Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 14);
Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15);
Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 16);
Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17); and
Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 18).

26. The method of claim 22 for inhibiting growth of Salmonella typhi comprising administering a growth inhibiting effective concentration of a polypeptide selected from the group consisting of:
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 7);
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Ala-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 8);
Gly-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 9);
Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 10);
Ser-Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 11);
Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 12);

Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13);
Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 14);
Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15);
Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 16); and
Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17).

27. The method of claim 22 for inhibiting growth of Yersinia enterocolitica comprising administering a growth inhibiting effective concentration of a polypeptide selected from the group consisting of:
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 7);
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Ala-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 8);
Gly-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 9);
Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 10);
Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 12); and
Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 14).

28. The method of claim 22 for inhibiting growth of Campylobacter jejuni comprising administering a growth inhibiting effective concentration of a polypeptide selected from the group consisting of:
Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13);
Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15);
Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-

His-Pro-Arg-Leu (SEQ ID NO: 16);
Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17); and
Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 18).

29. The method of claim 22 for inhibiting growth of Acinetobacter calcoaceticus comprising administering a growth inhibiting effective concentration of a polypeptide selected from the group consisting of:
Gly-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 9);
Ser-Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 11);
Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 12);
Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13);
Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 14);
Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15);
Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 16);
Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17); and
Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 18).

30. The method of claim 22 for inhibiting growth of Agrobacterium tumefaciens comprising administering a growth inhibiting effective concentration of a polypeptide selected from the group consisting of:
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 7);
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Ala-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 8);

Gly-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 9);
Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 12);
Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13);
Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 14);
Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15);
Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 16);
Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17); and
Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 18).

31. The method of claim 22 for inhibiting growth of Francisella tularensis or Haemophilus influenzae comprising administering a growth inhibiting effective concentration of a polypeptide selected from the group consisting of:
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 7);
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Ala-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 8);
Gly-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 9);
Ser-Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 11);
Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 12);
Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13);
Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 14);
Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-

Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15);
Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 16);
Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17); and
Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 18).

32. The method of claim 22 for inhibiting growth of Legionella pneumophila comprising administering a growth inhibiting effective concentration of a polypeptide selected from the group consisting of:
Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13);
Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15);
Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 16);
Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17); and
Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 18).

33. The method of claim 22 for inhibiting growth of Rhizobium meliloti comprising administering a growth inhibiting effective concentration of a polypeptide selected from the group consisting of:
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 7);
Asn-Arg-Pro-Thr-Tyr-Val-Pro-Ala-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 8);
Gly-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 9);
Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 10);
Asn-Lys-Pro-Arg-Pro-Gln-Gln-Val-Pro-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 12);

Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13); Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 14); Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15); Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 16); Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17); and Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 18).

34. A pharmaceutical composition comprising an antibacterial effective amount of the polypeptide of claim 1 and a pharmaceutically acceptable carrier.

35. A method for treating a subject infected with a bacterium comprising administering to the subject an antibacterial effective amount of a polypeptide of claim 1, thereby treating the subject.

36. A method for obtaining a purified apidaecin-like polypeptide from a Hymenopteran insect comprising

obtaining a sample of lymph from the insect;

treating the sample so as to obtain supernatant;

applying the supernatant to a reversed-phase high performance liquid chromatography column;

eluting from the column;

collecting the fractions eluted from the column; and

determining a fraction which contains the polypeptide, thereby obtaining the polypeptide from the insect.

37. The method of claim 36 wherein the obtaining a

sample of lymph comprises puncturing the abdomen of the insect and collecting the hemolymph.

38. The method of claim 36 wherein the treating comprises centrifuging.

39. The method of claim 36 wherein the eluting is eluting with an ascending acetonitrile gradient.

40. The method of claim 36 wherein the determining is determining by enzyme-linked immunoassay.

41. The method of claim 40 wherein the enzyme-linked immunoassay comprises a first antibody being anti-honeybee-apidaecin antibody.

42. The method of claim 36 further comprising, before obtaining a sample of insect lymph, immuno-inducing the insect.

43. The method of claim 42 wherein the immuno-inducing comprises infecting the insect with an immune-response-inducing effective amount of bacterium.

44. The method of claim 43 wherein the bacterium is E. coli.

45. A purified polypeptide having antibacterial activity comprising

a first sequence, at least seven amino acid residues are the same as Pro-Arg-Pro-Pro-His-Pro-Arg-X1 (SEQ ID NO: 1), wherein X1 is Ile or Leu;

a third sequence X2-Pro-X3-X4-X5-Pro (SEQ ID NO: 2), wherein

X2 is Arg or Lys,

X3 is Thr, Gln or Arg,

X4 is Tyr, Gln or Pro, and
X5 is Val or Ala,
the third sequence is N-terminal to the first sequence; and

a fourth sequence comprising at least five amino acid residues, at least one-third of the residues are Pro, the fourth sequence is N-terminal to the third sequence.

46. The polypeptide of claim 45 comprising up to about thirty-five amino acid residues.

47. The polypeptide of claim 45 wherein the first sequence is separated from the third sequence by up to two amino acid residues.

48. The polypeptide of claim 45 wherein the third sequence is separated from the fourth sequence by up to three amino acid residues.

49. The polypeptide of claim 45 wherein the first sequence is
Pro-Arg-X6-Pro-His-Pro-Arg-X1 (SEQ ID NO: 19), wherein X6 is an amino acid residue.

50. The polypeptide of claim 49 wherein X6 is Pro.

51. The polypeptide of claim 49 wherein X6 is Thr.

52. The polypeptide of claim 45, the fourth sequence comprising at least 13 amino acid residues.

53. The polypeptide of claim 45, the fourth sequence wherein at least one of every three consecutive amino acid residues is Pro.

54. The polypeptide of claim 45, the fourth sequence

comprising Pro-Arg-Pro.

55. The polypeptide of claim 45, the fourth sequence is selected from the group consisting of
Ser-Gln-Pro-Arg-Pro-Gln-Pro (SEQ ID NO: 20),
Gln-Val-Pro-Ile-Arg-Pro-Ser-Gln-Pro-Arg-Pro-Gln-Pro (SEQ ID NO: 21), and
Ser-Arg-Pro-Ser-Pro-Gln-Val-Pro-Ile-Arg-Pro-Ser-Gln-Pro-Arg-Pro-Gln-Pro (SEQ ID NO: 22).

56. DNA encoding the polypeptide of claim 45.

57. A purified antibody which binds the polypeptide of claim 45.

58. The antibody of claim 57 wherein the antibody is a polyclonal antibody.

59. A method for obtaining the purified antibody of claim 57 comprising:
coupling an antigen selected from the group consisting of honeybee apidaecin and the polypeptide of claim Q1 to a carrier protein,
immunizing a mammal with the coupled antigen; and
isolating the antibody from the mammal, thereby obtaining the purified antibody.

60. The method of claim 59 wherein the carrier protein is tuberculin purified protein derivative.

61. The method of claim 59 wherein the immunizing is immunizing by injecting.

62. The method of claim 59 wherein the mammal is a rabbit.

63. A method for determining the presence of the polypeptide of claim 45 in a sample comprising
incubating the sample with the antibody of claim 57, and
detecting an antibody-antigen complex,
thereby determining the presence of the polypeptide in the sample.

64. The method of claim 63 wherein the detecting comprises detecting by enzyme-linked immunoassay.

65. A method for inhibiting growth of a bacterium comprising exposing the bacterium to a growth inhibiting effective concentration of the polypeptide of claim 45.

66. A pharmaceutical composition comprising an antibacterial effective amount of the polypeptide of claim 45 and a pharmaceutically acceptable carrier.

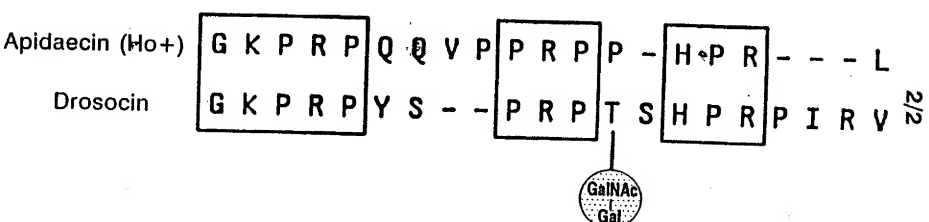
67. A method for treating a subject infected with a bacterium comprising administering to the subject an antibacterial effective amount of a polypeptide of claim 45, thereby treating the subject.

68. A method of claim 22 for inhibiting growth of Helicobacter pylori comprising administering a growth inhibiting effective concentration of a polypeptide selected from the group consisting of:
Gly-Lys-Pro-Asn-Arg-Pro-Arg-Pro-Ala-Pro-Ile-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 13);
Gly-Lys-Pro-Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 15);
Asn-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 16);
Gly-Lys-Pro-Ser-Lys-Pro-Arg-Pro-Ala-Pro-Ile-Lys-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (SEQ ID NO: 17); and

FIGURE 1

		1 2 a b c	3	5	7	9 a b	10	13	15	18	[MH+] calculated	[m/z] obtained
Honey bee	Hb Ia	G N - -	N	R P	V	Y I	P	- Q	P R P P H P R	I	2109.46	n.d.
	Hb Ib	. . . .	.	. .	.	. .	.	. .	. . . . .	L	2109.46	2110.0
	Hb II	. . . .	.	I .	.	. .	-	. .	. . . . .	.	2123.48	n.d.
	Hb III	. . . .	.	. .	.	. S	-	. .	. . . . .	.	2099.42	n.a.
Bumble bee	Bb +A	A - - -	N	R P	T	Y V	P	- P	P R P P H P R	L	1978.36	1978.5
	Bb -A	. . . .	.	. .	.	. .	-	. .	. . . . .	.	1907.28	1907.8
Cicada Killer	Ck P		N	R P	T	Y V	P	- P	P R P P H P R	L	1894.22	1894.0
	Ck A		.	. .	.	. .	-	A .	. . . . .	.	1869.19	1868.2
Bald-faced hornet	Ho +	G - K P	-	R P	Q	Q V	P	- -	P R P P H P R	L	1958.33	1960.0
	Ho -	. . . .	.	. .	.	. .	-	- -	. . . . .	.	1675.99	n.a.
Yellow jacket & german wasp	Yj +S	S N K P	-	R P	Q	Q V	P	- -	P R P P H P R	L	2102.46	2102.9
	Yj -S	. . . .	-	. .	.	. .	-	- -	. . . . .	.	2015.38	2015.8
C. disparis	Cd 1+	G - K P	N	R P	A	P I	Q	P R P P H P R		L	2282.72	2281.4
	Cd 1-	. . . .	.	. .	.	. .	.	. . . . .		.	2000.38	2001.1
	Cd 2+	. - . .	.	K .	.	. .	K	. . . . .		.	2254.75	2255.3
	Cd 2-	. . . .	.	K .	.	. .	K	. . . . .		.	1972.40	n.a
	Cd 3+	. - . .	S	K .	.	. .	K	. . . . .		.	2227.72	2227.8
	Cd 3-	. . . .	S	K .	.	. .	K	. . . . .		.	1945.38	1945.3
Conserved Sequence			R K	P		P		P R P P H P R		I L		

FIGURE 2


Apidaecin (Ho+)
G K P R P Q Q V P P R P P - H P R - - - L
Drosocin
G K P R P Y S - - P R P T S H P R P I R V
GalNAc
Gal

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/02626

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/86; 514/12, 13, 14, 21; 530/300, 324, 325, 326, 327, 387.1, 412, 417; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog (Medline, BIOSIS, Derwent WPI)
Search terms: apidaecin, peptide, antibacterial, bactericidal, antibiotic, wasp, hornet, bee, honeybee

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P ------ Y, P	Journal of Biological Chemistry, Vol. 269, No. 42, issued 21 October 1994, Casteels et al., "Biodiversity of apidaecin-type peptide antibiotics", pages 26107-26115, see the entire document.	1-11, 13, 16-32, 36-55, 59-65, 68 --- 12, 14, 15, 33-35, 56-58, 66, 67
A, P	US, A, 5,300,629 (CASTEELS ET AL.) 05 APRIL 1994, see the entire document.	1-68
A	Annual Review of Microbiology, Vol. 41, issued 1987, Boman et al., "Cell-free immunity in insects", pages 103-126, see the entire document.	1-68

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 JUNE 1995	08JUN1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ERIC GRIMES Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Research in Immunology, Vol. 141, issued 1990, Casteels, "Possible applications of insect antibacterial peptides", pages 940-942, see the entire document.	1-68
A	Biochemical and Biophysical Research Communications, Vol. 165, No. 2, issued 15 December 1989, Craig et al., "Mass spectrometric identification of peptides present in immunized and parasitised hemolymph from honeybees without purification", pages 637-643, see the entire document.	1-68
A	Biochemical and Biophysical Research Communications, Vol. 199, No. 1, issued 28 February 1994, Casteels et al., "*Apidaecin*-type peptide antibiotics function through a non-poreforming mechanism involving stereospecificity", pages 339-345, see the entire document.	1-68

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/02626

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A01N 63/02; A61K 38/10, 38/17; C07K 1/16, 4/12, 7/08, 14/435, 16/18; C12N 15/12; G01N 33/68

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/86; 514/12, 13, 14, 21; 530/300, 324, 325, 326, 327, 387.1, 412, 417; 536/23.5